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ORIGINAL ARTICLE

Effect of the NO donor "sodium nitroprusside" (SNP), the ethylene inhibitor "cobalt chloride" ($CoCl_2$) and the antioxidant vitamin E "α-tocopherol" on in vitro shoot proliferation of *Sideritis raeseri* Boiss. & Heldr. subsp. *raeseri*

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Abstract In the present study, the effects of the NO donor "sodium nitroprusside" (SNP) and the ethylene inhibitor "$CoCl_2$" combined with 2.2 μM 6-benzyladenine (BA), and the antioxidant vitamin E "α-tocopherol" in combination with 2.2 μM zeatin (ZT) and 0.2 μM α-naphthaleneacetic acid (NAA) on in vitro shoot proliferation of *Sideritis raeseri* Boiss. & Heldr. subsp. *raeseri*, one of the seven endemic mountain tea species of the Greek flora were investigated. The basal nutrient culture medium used in all experiments was the MS (Murashige, Skoog, Physiol Plant 15:473–497, 1962). Taking simultaneously into consideration all shoot proliferation parameters evaluated, the explants performance was optimum and significantly better among others when treated with 2.2 μM BA + 10 μM SNP or 2.2 μM BA + 2.5 μM $CoCl_2$, or 2.2 μM ZT + 0.2 μM NAA + 11.6 μM α-tocopherol. In particular, 10 μM SNP gave 4.6 ± 0.6 shoots/explant and 100% shoot multiplication percentage while 20 μM SNP exhibited the greatest shoot length (17.5 ± 1.8 mm). However, SNP (10–40 μM) caused hyperhydricity symptoms to the 30–100% of explants. $CoCl_2$ applied at 2.5 μM led to the production of 5.5 ± 0.7 shoots/explant, 11.8 ± 1.1 mm shoot length and 100% shoot multiplication percentage and concurrently decreased the percentage of vitrified explants from 33.3 to 20%. 11.6 μM α-tocopherol gave 6.7 ± 0.8 shoots/explant, 14.5 ± 0.7 mm shoot length, 100% shoot multiplication percentage, diminishing vitrification percentage from 41.6 to 8.3% (33.3% decrease) and completely inhibiting the appearance of necrotic explants, indicating its antioxidant properties as a vitamin. Shoot-tip explants responded better and exhibited better shoot proliferation results when cultured in MS medium with 2.5 μM $CoCl_2$ for 5 weeks or 11.6 μM α-tocopherol for 9 weeks rather than with SNP for 4 weeks. In this study, three new different protocols were established for the in vitro shoot proliferation stage of *S. raeseri*.

Keywords α-Tocopherol · Cobalt chloride · Hellenic mountain tea · In vitro shoot proliferation · Sodium nitroprusside · *Sideritis raeseri*

Introduction

Among the threatened wild-growing species in Greece are those of the genus *Sideritis* (Lamiaceae). Taxonomic revision (Papanikolaou and Kokkini 1982) of *Sideritis* L. section Empedoclia, reported the following seven species of the genus *Sideritis* as part of the Greek flora: *S. raeseri* Boiss. & Heldr., *S. clandestina* Chaub. & Borry, *S. scardica* Griseb., *S. euboea* Heldr., *S. athoa* Papanikolaou & Kokkini, *S. perfoliata* L. and *S. syriaca* L. From the various species of *Sideritis* only *S. raeseri*, which is the most widespread, is systematically cultivated in Greece. In particular, *S. raeseri* is distributed in central, north-central and northern parts of the country, typically in the range of Pindos (Strid and Tan 1991) and it self-sows in Parnassus, Timfristos (Velouchi) and other mountains of Aetolia, Doris and Fthiotida (Goliaris 1984).

Conservation of endemic, endangered (IUCN 2001), medicinal and aromatic plants including *Sideritis* genus native in Greece is beyond regional scope and becomes of global significance. *Sideritis* species are of great

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economic importance which is not only related to their use as a spice. In fact, as recent studies have pointed out, *Sideritis* species are traditionally used in many other ways as their essential oils have antimicrobial, antifungicidal, cytotoxic, antiviral, nematicidal and antioxidant properties (Gonzalez-Burgos et al. 2011; Todorova and Trendaflova 2014; Yigıt 2014). Seeds of *Sideritis* species collected from native populations exhibit dormancy and have a low germination ability under laboratory and natural conditions (about 5%) (Evstatieva and Koleva 2000). Micropropagation is more complicated and sophisticated than traditional methods, requires special training, the initial costs for setting up a micropropagation unit are relatively high and special laboratory equipment is needed, as well as expensive and very pure chemicals (Cachiţă-Cosma 1987). However, micropropagation method was considered necessary for the mass vegetative propagation of *S. raeseri* as the nature of plants belonging to *Sideritis* genus is such that their asexual propagation with cuttings results in low rooting percentages and losses during acclimatization stage. Tissue culture of *Sideritis* species has been reported on several occasions (Sanchez-Gras and Segura 1987, 1988, 1997; Garcia-Granados et al. 1994; Faria et al. 1998; Erdağ and Yórekli 2000; Uçar and Turgut 2009; Papafotiou and Kalantzis 2009; Danova et al. 2013). In vitro culture of *Sideritis* spp. as an approach for conservation of the natural habitats and provision of herb with high and permanent quality has also been important in order to increase the accumulation of biologically active compounds and obtain herb with permanent and good quality (Gonzalez-Burgos et al. 2011; Todorova and Trendaflova 2014). Therefore, in vitro propagation is a suitable method for plant regeneration, micropropagation and long-term storage of virus-free plant material in a commercial scale, preventing the destruction of the flora, in comparison with traditional propagation systems including seed, cutting, grafting, air-layering etc. (Garcia-Gonzales et al. 2010).

Sodium nitroprusside (SNP) releases nitric oxide (NO), a highly reactive gas and a ubiquitous bioactive molecule which plays a central role in signal transduction in plant stress response (Arasimowicz and Wieczorek 2007). The effects of NO on different types of cells indicate that NO is a potent oxidant or an effective antioxidant (Qiao and Fan 2008). SNP releases NO in a pH dependent manner that promotes plant growth and development and reduces senescence (Kolberz et al. 2008). NO produced by SNP has currently been considered a new member of phytohormones (Leterrier et al. 2012) and may scavenge other reactive intermediaries like reactive oxygen species (ROS) (Laspina et al. 2005). NO synthesis was induced by cytokinins in tobacco, parsley and *Arabidopsis* cell cultures (Tun et al. 2001).

Accumulation of ethylene in culture vessels may induce growth abnormalities in plants generated in vitro including inhibition of growth, leaf epinasty, leaf senescence and diminution of foliar area (Steinitz et al. 2010). However, the influence of ethylene in plant cells and tissues grown in vitro is diverse and often controversial depending on plant species and even the cultivar (Jha et al. 2007). The addition of ethylene inhibitors such as cobalt chloride ($CoCl_2$) to culture media have been demonstrated to improve regeneration and growth performance in both dicot and monocot plant tissue cultures (Sandra and Maira 2013). Cobalt is an inorganic micronutrient used in most plant tissue culture media based on level established by Murashige and Skoog (1962) for tobacco tissue culture. The low level of cobalt ion promotes growth factors such as plant height, number of leaves per plant as well as fresh and dry weight of leaves and roots (Hilmy and Gad 2002). Cobalt increases the growth of seedlings and alleviates the senescence of aged tissues, inhibiting the activities of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase and thus reducing ethylene production (Li et al. 2005). The promoting effect of cobalt can be ascribed to its role in several physiological activities like growth, photosynthesis and respiration (El-Sheekh et al. 2003; Aziz Eman et al. 2007).

Tocopherols (α-, β-, γ-, and δ-tocopherol) represent a group of lipophilic antioxidants that are synthesized only by photosynthetic organisms. It is widely believed that the main functions of tocopherols are protection of pigments and proteins of photosystem and polyunsaturated fatty acids from oxidative damage caused by reactive oxygen species (Woo et al. 2014). α-Tocopherol (vitamin E) is a common term used for tocopherols and tocotrienols. It is an important antioxidant that is directly involved in scavenging oxygen free radicals and quenching lipid peroxidation chain reactions that occur during oxidation reactions with polyunsaturated fatty acids (Sattler et al. 2004). α-Tocopherol reactions result in the formation of tocopheroxyl radicals that react with other antioxidants to regenerate the active molecule (Liebler 1993; Carelli et al. 2005). Regarding its role in plant tissue culture systems, vitamin E improved in vitro recovery of cryopreserved blackberry shoot tips (Uchendu and Reed 2010). Using sunflower cell cultures, a suitable in vitro production system of natural α-tocopherol was established (Caretto et al. 2004). In addition, there was a substantial improvement in somatic embryogenesis of Loblolly pine (*Pinus taeda* L.) in the early-stage of somatic embryo growth by supplementing the medium with vitamin E (Pullman et al. 2006). According to Woo et al. (2014) the expression of tobacco tocopherol cyclase in rice regulated antioxidative defense and drought tolerance.

The objectives of this study were to develop and establish three optimal protocols for in vitro clonal shoot multiplication of *S. raeseri*, an endemic to Greek flora species.

There is no experimental evidence available that directly shows the effect of SNP, $CoCl_2$ and α-tocopherol on in vitro shoot proliferation of *Sideritis* spp. Therefore, this research study focused on investigating the exogenous effect of these three agents on shoot proliferation of *S. raeseri* under in vitro conditions.

Materials and methods

For the initial establishment of the plant material in vitro, shoot tips of *S. raeseri*, 1.5-2 cm long were cut and removed from mother plants maintained in a peat:perlite (1:1) substrate in pots under unheated-greenhouse conditions. For the disinfection of the experimental plant material, shoot tip explants were soaked in 70% ethanol for 1 min followed by 2% NaOCl solution for 15 min under continuous stirring and then washed into sterile distilled water for 4–5 times. The successfully established explants (pathogen-free) were sub-cultured every 4 weeks until a sufficient amount of plant material was obtained. The nutrient culture medium used for all experiments was the MS (Murashige and Skoog 1962).

Three experiments were conducted aiming to enhance in vitro shoot proliferation of *S. raeseri* shoot tip explants. In the first experiment, SNP (Fluka Biochemika) was exogenously applied at 4 concentrations (0, 10, 20, 40 μM) in combination with 2.2 μM BA (Sigma–Aldrich). In the second experiment, $CoCl_2$ $6H_2O$ (Sigma–Aldrich) was tested at 9 concentrations (0.1-contol, 0.5, 1, 2.5, 5, 10, 25, 50, 100 μM) in combination with 2.2 μM BA. The normal concentration of $CoCl_2$ $6H_2O$, which is an essential microelement of MS culture medium is 0.1 μM (=0.025 mg/l) (Murashige and Skoog 1962), thus, this concentration was considered the control treatment of this experiment. In the third experiment, α-tocopherol (Sigma–Aldrich) was added to culture medium at five concentrations (0, 2.3, 5.8, 11.6, 23.2 μM) in combination with 2.2 μM zeatin (ZT; Sigma–Aldrich) and 0.2 μM α-naphthaleneacetic acid (NAA; Sigma–Aldrich). For all three experiments, the MS culture medium was also supplemented with 30 g/l sucrose (Duchefa Biochemie). In the first and second experiments with SNP and $CoCl_2$, respectively, 3 g/l of Phytagel (Sigma–Aldrich) was used as gelling agent while 3 g/l of Gelrite (Duchefa Biochemie), another type of gelling agent was applied for the third experiment with α-tocopherol. The pH of the culture medium in all three experiments was adjusted to 5.8 before adding the gelling agent and afterwards the medium was sterilized at 121 °C for 20 min. Shoot-tip explants were transferred for the first and second experiments with SNP and $CoCl_2$, respectively into flat-base glass test tubes of 25 × 100 mm containing 10 ml of MS medium and for the third experiment with α-tocopherol into Magenta vessels containing 35 ml of MS medium. All cultures were maintained in a growth chamber. The chamber was programmed to maintain 16-h light duration (40 μmol/m^2/s) supplied by cool white fluorescent lamps and a constant temperature of 22 ± 2 °C. After 4 weeks of culture for the first experiment with SNP, 5 weeks for the second experiment with $CoCl_2$ and 9 weeks for the third experiment with α-tocopherol, measurements were taken regarding shoot proliferation characteristics including shoot number/explant, shoot length (mm), shoot multiplication percentage (i.e. number of initial explants able to proliferate new vegetative masses/total number of initial explants × 100%), callus formation, vitrification and necrosis percentages (%).

Statistical analysis

All three experiments were repeated twice and the reported data are the means of the two comparisons, separately for each experiment. The first experiment with SNP included 4 treatments with 20 replicates/treatment in total (10 explants were used the first time and other 10 explants were used the second time in the repetition of the experiment) and one shoot-tip explant placed in each test tube. The second experiment with $CoCl_2$ included 9 treatments with 20 replicates/treatment in total (10 explants the first time and other 10 explants the second time in the repetition of the experiment) and one shoot-tip explant placed in each test tube. The third experiment with α-tocopherol included 5 treatments with 24 replicates/treatment in total (12 explants the first time and other 12 explants the second time in the repetition of the experiment) and 4 shoot-tip explants placed in each Magenta vessel. Each experiment was completely randomized and analyzed with 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 ± SE in order significant differences among the treatments to be established.

Results

Experiment 1: effect of SNP on in vitro shoot proliferation of *S. raeseri*

The combined effect of 10 μM SNP + 2.2 μM BA positively affected shoot proliferation in terms of shoot number/explant (4.6 ± 0.6) and shoot multiplication percentage (100%) in relation to the individual effect of 2.2 μM BA (3.2 ± 0.6 shoots/explant and 70% shoot multiplication percentage) (Table 1). However, SNP (10–40 μM) in combination with 2.2 μM BA gave similar shoot length results (14.7 ± 0.9–17.5 ± 1.8 mm) (Fig. 1b–d) to the

Table 1 Effect of SNP concentration (0–40 µM) combined with 2.2 µM BA on shoot number/explant, shoot length (mm), shoot multiplication, callus induction and vitrification percentages (%) in *Sideritis raeseri* Boiss. & Heldr. subsp. *raeseri* after 4 weeks of culture

Treatments (µM)	Shoot number/explant	Shoot length (mm)	Shoot multiplication percentage (%)	Callus induction percentage (%)	Vitrification percentage (%)
2.2 BA + 0 SNP (control)	3.2 ± 0.6ab	16.7 ± 1.6a	70b	90b	0a
2.2 BA + 10 SNP	4.6 ± 0.6c	14.7 ± 0.4a	100c	100c	40c
2.2 BA + 20 SNP	2.4 ± 0.4ab	17.5 ± 1.8a	70b	90b	30b
2.2 BA + 40 SNP	1.2 ± 0.2a	15.5 ± 0.9a	10a	0a	100d
P values	0.000***	0.494 ns	0.000***	0.000***	0.000***

The experiment was repeated twice with n = 20 replications/treatment in total (10 replicates/treatment the first time the experiment conducted and other 10 replicates the second time). Means ± SE denoted by the same letter in each column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$

ns $P \geq 0.05$, ***$P \leq 0.001$

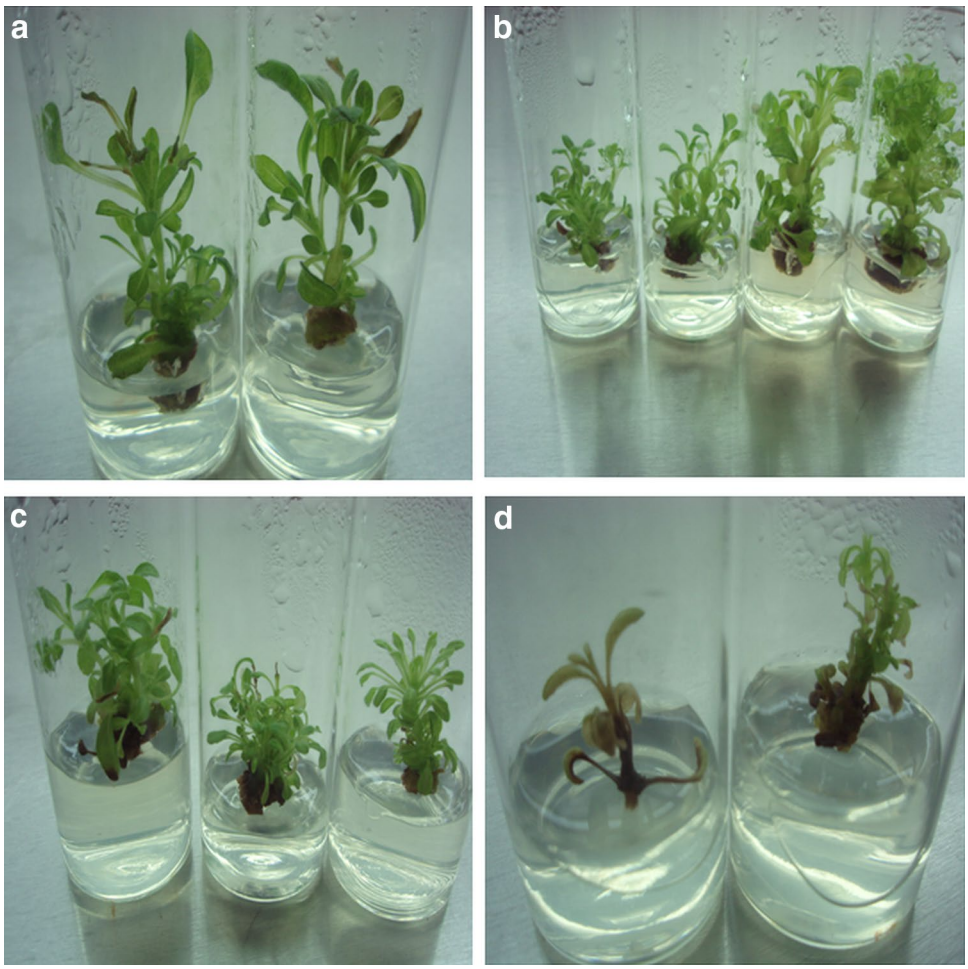

Fig. 1 Effect of the concentration of the NO donor "SNP" combined with 2.2 µM BA on in vitro shoot proliferation of *Sideritis raeseri* Boiss. & Heldr. subsp. *raeseri*: (**a**) control (2.2 µM BA, SNP-free), (**b**) 2.2 µM BA + 10 µM SNP, (**c**) 2.2 µM BA + 20 µM SNP, (**d**) 2.2 µM BA + 40 µM SNP

control (16.7 ± 1.6 mm) (Fig. 1a). The highest applied SNP concentration of 40 µM was toxic and resulted in a significant reduction in shoot number and shoot multiplication percentage, caused extensive hyperhydricity to the 100% of explants and completely inhibited callus formation at the base of the explants. Vitrification percentage was increased with an increase in SNP concentration. No vitrified explants were observed in the control treatment (2.2 µM BA, SNP-free).

Experiment 2: effect of $CoCl_2$ on in vitro shoot proliferation of *S. raeseri*

2.5 μM and 10–50 μM $CoCl_2$ had a positive effect on shoot number/explant, 5.5 ± 0.7 and 5.3–5.4 respectively, in relation to the control (2.6 ± 0.6 shoots/explant) (Table 2). However, 0.5–100 μM $CoCl_2$ + 2.2 μM BA did not promote nor inhibited the elongation of microshoots (10.6 ± 0.9–16.0 ± 1.5 mm) (Fig. 2b–i) significantly, compared with the control (0.1 μM $CoCl_2$ + 2.2 μM BA/14.2 ± 0.8 mm) (Fig. 2a). Shoot multiplication percentage was significantly raised (87.5–100%) when explants were exposed to 0.5–100 μM $CoCl_2$ with respect to the control (55.5%). $CoCl_2$ (0.5–1 μM and 5–100 μM) intensified the problem of vitrified explants (40–100%) compared to the control (33.3%), whereas 2.5 μM $CoCl_2$ diminished hyperhydricity percentage to 20%. Callus induction percentage was substantially reduced from 66.6% (control) to 20% when 100 μM $CoCl_2$ was added to the culture medium.

Experiment 3: effect of α-tocopherol (vitamin E) on in vitro shoot proliferation of *S. raeseri*

Best results in terms of shoot number/explant (6.7 ± 0.8) and shoot multiplication percentage (100%) were recorded in the combination treatment 2.2 μM ZT + 0.2 μM NAA + 11.6 μM α-tocopherol (Fig. 3d), compared with the control (3.4 ± 0.5 shoots/explant and 91.6% shoot multiplication percentage) (Table 3; Fig. 3a). However, shoot length was decreased by 0.8 cm, from 22.6 ± 2.5 mm (control) to 14.5 ± 0.7 mm when 11.6 μM α-tocopherol was added to the culture medium. α-Tocopherol concentrations lower (2.3 or 5.8 μM) and higher (23.2 μM) than 11.6 μM did not promote or suppress shoot elongation of explants (Fig. 3b, c, e) substantially. According to Table 4, no change in callus induction percentage (100%) was observed with respect to the control due to α-tocopherol application. The percentage of vitrified explants was diminished from 41.6% (control 2.2 μM ZT + 0.2 μM NAA) to 8.3% (33.3% decrease) by supplementing the culture medium with 11.6 μM α-tocopherol. The incorporation of 11.6 μM α-tocopherol into the culture medium led simultaneously to the production of microshoots with no apparent symptoms of necrosis (Table 4).

Discussion

Prior to this research work, there was available only one published protocol for in vitro shoot proliferation of *S. raeseri* conducted by Sarropoulou and Maloupa (2015) indicating the usefulness of MS medium supplemented with 2.2 μM BA, 0.05 μM indole-3-butyric acid (IBA), 0.05 μM NAA, 0.15 μM gibberellic acid (GA_3) and 740 μM glycine-betaine in combination with 25 μM dikegulac on multiple shoot regeneration. Therefore, in the present study, three new protocols have been developed for in vitro shoot proliferation of *S. raeseri*.

In the first experiment of the present study, the combined effect of 10 μM SNP + 2.2 μM BA stimulated in vitro shoot proliferation of *S. raeseri* explants in terms of shoot number and shoot multiplication percentage in

Table 2 Effect of $CoCl_2$ concentration (0.1–100 μM) combined with 2.2 μM BA on shoot number/explant, shoot length (mm), shoot multiplication, vitrification and callus induction percentages (%) in *Sideritis raeseri* Boiss. & Heldr. subsp. *raeseri* after 5 weeks of culture (MS medium contains 0.1 μM $CoCl_2$—control treatment)

Treatments (μM)	Shoot number/explant	Shoot length (mm)	Shoot multiplication percentage (%)	Vitrification percentage (%)	Callus induction percentage (%)
2.2 BA + 0.1 $CoCl_2$ (control)	2.6 ± 0.6a	14.2 ± 0.8abc	55.5a	33.3b	66.6b
2.2 BA + 0.5 $CoCl_2$	4.2 ± 0.5ab	10.6 ± 0.9a	100d	100g	100e
2.2 BA + 1 $CoCl_2$	3.4 ± 0.4ab	15.1 ± 2.4bc	90c	60f	90d
2.2 BA + 2.5 $CoCl_2$	5.5 ± 0.7b	11.8 ± 1.1abc	100d	20a	80c
2.2 BA + 5 $CoCl_2$	4.0 ± 0.4ab	16.0 ± 1.5c	87.5b	50e	62.5b
2.2 BA + 10 $CoCl_2$	5.4 ± 0.5b	14.2 ± 1.2abc	100d	40c	90d
2.2 BA + 25 $CoCl_2$	5.3 ± 0.7b	13.3 ± 1.4abc	88.8bc	44.4d	88.8d
2.2 BA + 50 $CoCl_2$	5.4 ± 0.9b	10.9 ± 0.8ab	100d	50e	90d
2.2 BA + 100 $CoCl_2$	4.3 ± 1.2ab	12.0 ± 0.9abc	90c	100g	20a
P values (one-way ANOVA)	0.079 ns	0.058 ns	0.000***	0.000***	0.000***

The experiment was repeated twice with n = 20 replications/treatment in total (10 replicates/treatment the first time the experiment conducted and other 10 replicates the second time). Means ± SE denoted by the same letter in each column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$

ns $P \geq 0.05$, ***$P \leq 0.001$

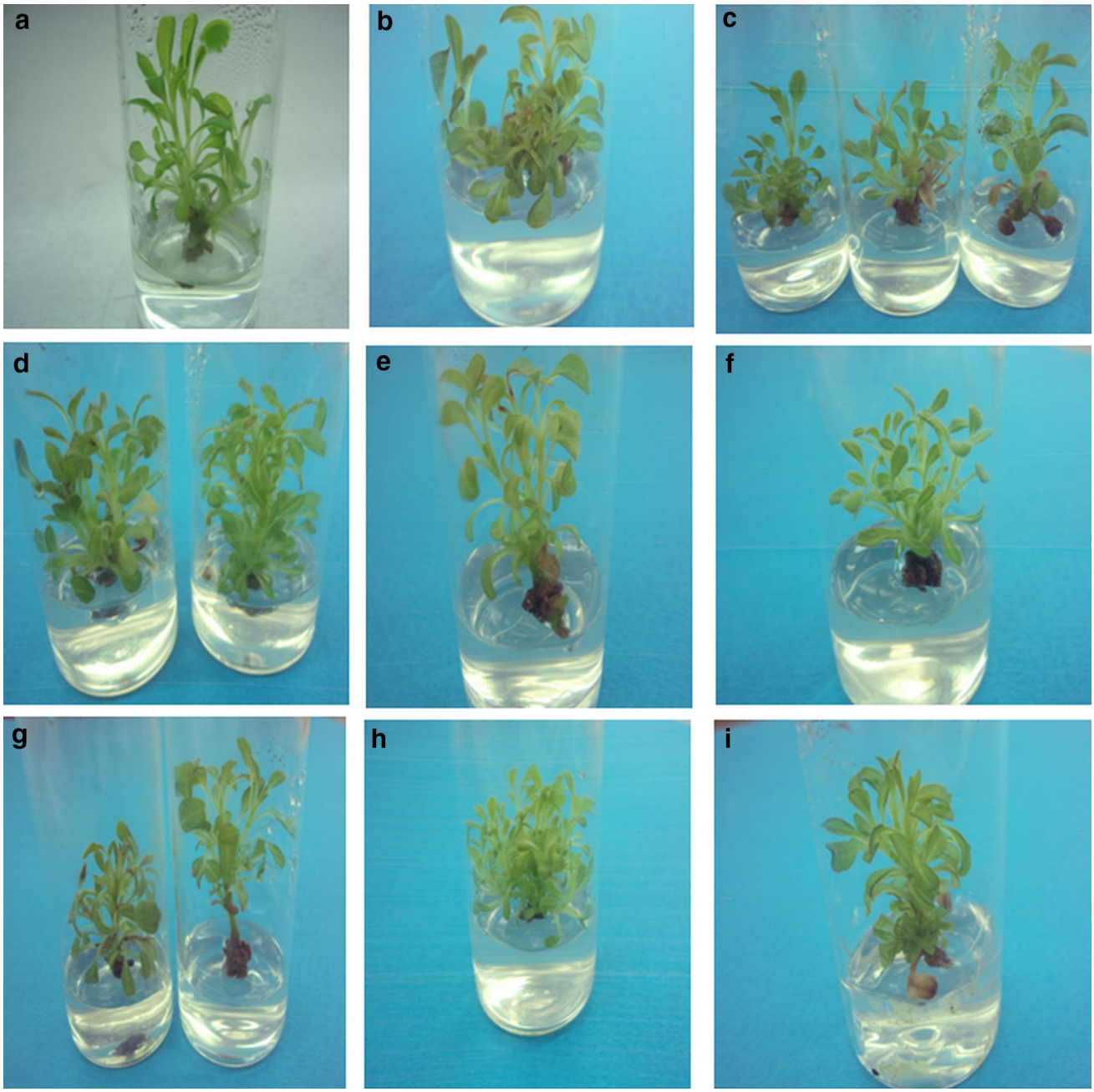

Fig. 2 Effect of the concentration of the ethylene inhibitor $CoCl_2$ combined with 2.2 µM BA on in vitro shoot proliferation of *Sideritis raeseri* Boiss. & Heldr. subsp. *raeseri*: (**a**) control (2.2 µM BA + 0.1 µM $CoCl_2$), (**b**) 2.2 µM BA + 0.5 µM $CoCl_2$, (**c**) 2.2 µM BA + 1 µM $CoCl_2$, (**d**) 2.2 µM BA + 2.5 µM $CoCl_2$, (**e**) 2.2 µM BA + 5 µM $CoCl_2$, (**f**) 2.2 µM BA + 10 µM $CoCl_2$, (**g**) 2.2 µM BA + 25 µM $CoCl_2$, (**h**) 2.2 µM BA + 50 µM $CoCl_2$, (**i**) 2.2 µM BA + 100 µM $CoCl_2$

comparison to the effect of BA solely, showing synergistic action between the NO donor SNP and the cytokinin BA. Our results are in agreement with those presented in the Gisela 6 (*Prunus cerasus* × *Prunus canescens*) cherry rootstock by Sarropoulou et al. (2015). Cytokinins induce a burst of NO in plant cell cultures, suggesting that NO is involved in cytokinin signal transduction (Tun et al. 2001). Our records are also in line with those reported by Han et al. (2009), who found that the multiplication of *Malus hupehensis* Rehd. var. *pinyiensis* Jiang plantlets was significantly promoted by applying 20 µM SNP combined with 2 µM BA and 1 µM ZT to MS medium. Carimi et al. (2005) found that the cytokinin BA induced NO accumulation in suspension cell cultures. The positive effect of SNP on in vitro shoot proliferation has also been reported for other plant species including *Albizzia*

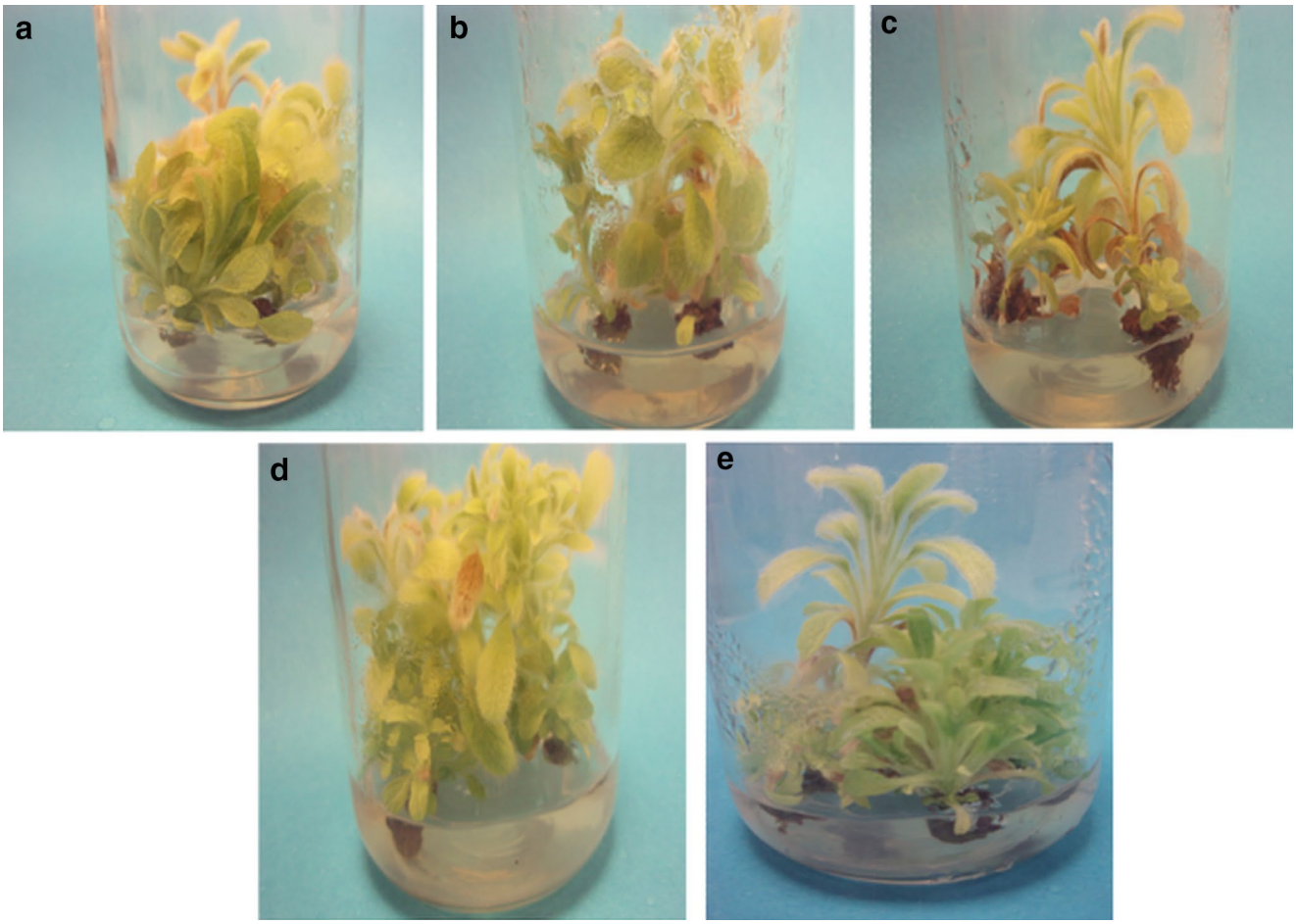

Fig. 3 Effect of the concentration of α-tocopherol (vitamin E) combined with 2.2 μM ZT and 0.2 μM NAA on in vitro shoot proliferation of *Sideritis raeseri* Boiss. & Heldr. subsp. *raeseri*: (**a**) Control (2.2 μM ZT + 0.2 μM NAA, α-tocopherol-free), (**b**) 2.2 μM ZT + 0.2 μM NAA + 2.3 μM α-tocopherol, (**c**) 2.2 μM ZT + 0.2 μM NAA + 5.8 μM α-tocopherol, (**d**) 2.2 μM ZT + 0.2 μM NAA + 11.6 μM α-tocopherol, (**e**) 2.2 μM ZT + 0.2 μM NAA + 23.2 μM α-tocopherol

Table 3 Effect of α-tocopherol (vitamin E) concentration (0–23.2 μM) combined with 2.2 μM zeatin (ZT) and 0.2 μM α-naphthaleneacetic acid (NAA) on shoot number/explant, shoot length (mm) and shoot multiplication percentage (%) in *Sideritis raeseri* Boiss. & Heldr. subsp. *raeseri* after 9 weeks of culture

Treatments (μM)	Shoot number/explant	Shoot length (mm)	Shoot multiplication percentage (%)
2.2 ZT + 0.2 NAA + 0 α-tocopherol (control)	3.4 ± 0.5a	22.6 ± 2.5b	91.6c
2.2 ZT + 0.2 NAA + 2.3 α-tocopherol	3.5 ± 0.5a	19.1 ± 2.2ab	100d
2.2 ZT + 0.2 NAA + 5.8 α-tocopherol	2.6 ± 0.5a	21.9 ± 4.0ab	83.3a
2.2 ZT + 0.2 NAA + 11.6 α-tocopherol	6.7 ± 0.8b	14.5 ± 0.7a	100d
2.2 ZT + 0.2 NAA + 23.2 α-tocopherol	3.3 ± 0.5a	20.4 ± 1.7ab	87.5b
P values (one-way ANOVA)	0.000***	0.179 ns	0.000***

The experiment was repeated twice with n = 24 replications/treatment in total (12 replicates/treatment the first time the experiment conducted and other 12 replicates the second time). Means ± SE denoted by the same letter in each column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$

ns $P \geq 0.05$, ***$P \leq 0.001$

Table 4 Effect of α-tocopherol (vitamin E) concentration (0–23.2 μM) combined with 2.2 μM zeatin (ZT) and 0.2 μM α-naphthaleneacetic acid (NAA) on callus induction, vitrification and necrosis percentages (%) in *Sideritis raeseri* Boiss. & Heldr. subsp. *raeseri* after 9 weeks of culture

Treatments (μM)	Callus induction percentage (%)	Vitrification percentage (%)	Necrosis percentage (%)
2.2 ZT + 0.2 NAA + 0 α-tocopherol (control)	100a	41.6c	25c
2.2 ZT + 0.2 NAA + 2.3 α-tocopherol	100a	50d	16.6b
2.2 ZT + 0.2 NAA + 5.8 α-tocopherol	100a	50d	16.6b
2.2 ZT + 0.2 NAA + 11.6 α-tocopherol	100a	8.3a	0a
2.2 ZT + 0.2 NAA + 23.2 α-tocopherol	100a	37.5b	25c
P values (one-way ANOVA)	1.000 ns	0.000***	0.000***

The experiment was repeated twice with n = 24 replications/treatment in total (12 replicates/treatment the first time the experiment conducted and other 12 replicates the second time). Means ± SE denoted by the same letter in each column are not statistically significant different from each other according to the Duncan's multiple range test at $P \leq 0.05$

ns $P \geq 0.05$, ***$P \leq 0.001$

lebbeck L. (Kalra and Babbar 2012), *Linum usitatissimum* L. (Kalra and Babbar 2010), *Dioscorea opposite* Thunb (Xu et al. 2009), *Triticum aestivum* L. (Tian and Lei 2007), *Kosteletzkya virginica* L. (Guo et al. 2009) and *Brassica napus* L. (Jhanji et al. 2012). These results indicate that NO may interact with auxin and cytokinin, linking the regulation of cell division to differentiation during de-differentiation and re-differentiation of plant cells (Otvos et al. 2005). Therefore, the supplementation of the MS culture medium with the NO-donor SNP improved the in vitro shoot proliferation potential and efficiency of *S. raeseri*. In the CAB-6P (*Prunus cerasus* L.) cherry rootstock, on the contrary, the combined effect of 10–50 μM SNP + 4.4 μM BA suppressed shoot proliferation of shoot-tip explants cultured in vitro regarding shoot number and multiplication percentage (Sarropoulou et al. 2015).

In the current study, SNP did not enhance nor suppress shoot elongation of *S. raeseri* explants. Similarly, in CAB-6P and Gisela 6 cherry rootstocks, the combined effect of BA + SNP did not differentiate shoot length significantly compared to the individual effect of BA (Sarropoulou et al. 2015). In contrast, shoot length of purslane (*Portulaca oleracea* L.) seedlings treated with 100 μM NO was significantly decreased under non-stress conditions (Fendereski et al. 2015). NO (precursor of SNP) has been reported to influence several plant developmental events in which gibberellins (GAs) play crucial roles such as seed germination, hypocotyl elongation, acquisition of photomorphogenic traits and others (Beligni and Lamattina 2000). However, so far, the actual interaction between NO and GAs has been described for only a limited number of these physiological events. In fact, the already known mechanisms underlying the interplay between GAs and NO are restricted to the inhibition of hypocotyl elongation during seedling de-etiolation (Leon and Lozano-Juste 2011). NO has been described to act upstream of GAs (Bethke et al. 2007) regulating both biosynthesis and perception/transduction of GAs (Leon and Lozano-Juste 2011).

In the present study employing *S. raeseri*, callus induction percentage was high (90–100%) in all SNP treatments including control except for 40 μM SNP where only the 10% of the explants induced callus. According to a previous study conducted by Sarropoulou et al. (2015) in CAB-6P and Gisela 6 cherry rootstocks, callus induction was evident only when SNP (0–50 μM) was applied with NAA and not when applied alone or in combination with BA. *Dioscorea opposite* Thunb tuber explants cultured in vitro, when treated with 40 μM SNP gave 87% callus induction percentage (Xu et al. 2009). Different results were exhibited by Han et al. (2009) who did not observe callus formation in *Malus hupehensis* explants treated with SNP.

$CoCl_2$ (0.5–100 μM) applied simultaneously with 2.2 μM BA enhanced in vitro shoot proliferation of *S. raeseri* explants increasing shoot number, shoot length and shoot multiplication percentage. According to Sarropoulou et al. (2016), $CoCl_2$ led to the production of multiple shoots in Gisela 6 cherry rootstock to a limiting degree, while it had absolutely no effect in the CAB-6P cherry rootstock. In consistency with our findings, the exogenous administration of 30 μM $CoCl_2$ to the culture medium resulted in the optimum response of explants regarding shoot number, shoot length and shoot multiplication percentage in several other plant species including pepper (*Capsicum frutescens* Mill.) (Sharma et al. 2008), cucumber (*Cucumber sativus* L.) (Vasudevan et al. 2006) and Gisela 6 cherry rootstock (*P. cerasus* × *P. canescens*) (Sarropoulou et al. 2016). The ethylene inhibitor $CoCl_2$ has been shown to be effective for shoot proliferation by inhibiting ethylene production in cucumber (Mhatre et al. 1998) and muskmelon (Yadav et al. 1996). According to Chae and Park (2012), 4.2 μM $CoCl_2$ promoted shoot proliferation of *Echinacea angustifolia* whereas higher $CoCl_2$ concentrations (21–84 μM) had an inhibitory effect. There is evidence which clearly shows

that Co interacts with other microelements of nutrient culture medium to form complexes with plant growth regulators, improving the metabolic activities that lead to shoot initiation and development (Palit et al. 1994). Another explanation for the beneficial effect of Co on plant growth could be ascribed according to Rathsooriya and Nagarajah (2003) to an increase in leaf water potential compared with Co-untreated plants, thus enhancing the process of photosynthesis.

In the present study, shoot length of *S. raeseri* explants was considerably enhanced due to $CoCl_2$ + BA application. In CAB-6P and Gisela 6 cherry rootstocks cultured in vitro, on the contrary, shoot length of explants was significantly diminished when 10–50 μM $CoCl_2$ was exogenously applied to the MS medium (BA-free) (Sarropoulou et al. 2016). Our results are also not in line with those presented for chickpea (*Cicer arietinum* cv. T-3), where $CoCl_2$ negatively affected shoot length (Khan and Khan 2010). Similar results to our study were reported for broccoli (*Brassica oleracea* var. *italica*), where the addition of different Co levels to the growth media significantly increased plant height (Gad and Abd El-Moez 2011). In greengram (*Vigna radiata* L. Wilczek), on the other hand, 210 μM $CoCl_2$ significantly increased shoot length whereas higher $CoCl_2$ concentrations resulted in a considerable decrease of shoot length (Abdul Jaleel et al. 2009). Co at high levels may inhibit shoot growth directly by inhibiting either cell division or cell elongation, or combination of both concurrently, resulting in limited exploration of culture medium volume for uptake and translocation of nutrients and water from plant tissues, and thus inducing mineral deficiency (Hemantaranjan et al. 2000).

In *S. raeseri*, 11.6 μM α-tocopherol combined with 2.2 μM ZT and 0.2 μM NAA promoted explants' in vitro shoot proliferation in terms of shoot number and shoot multiplication percentage. Our results are in agreement with those recorded by Gupta and Datta (2003) who noted that 100 μM α-tocopherol stimulated shoot organogenesis of *Gladiolus hybridus* Hort in vitro culture. According to Lee and Read (1988), in *Petunia hybrida* "Champagne" in vitro culture, the formation of adventitious shoots from leaf segments was accelerated due to the application of α-tocopherol (1.1–4.6 μM) + BA (0.8 μM) while the combined effect of α-tocopherol + NAA did not have any effect. In contrast, 11.6 μM α-tocopherol adversely affected shoot elongation of *S. raeseri* explants whereas lower (2.3–5.8 μM) and higher (23.2 μM) α-tocopherol concentrations did not differentiate shoot length considerably. However, shoot height of in vitro regenerated *P. hybrida* "Champagne" explants was not altered significantly while increasing α-tocopherol concentration for each NAA level (Lee and Read 1988).

In *S. raeseri*, the percentage of vitrified explants was remarkably decreased and the phenomenon of microshoots with symptoms of browning and subsequent necrosis was completely diminished (total disappearance) under the influence of 11.6 μM α-tocopherol, showing that α-tocopherol has ameliorating effects because of its antioxidant properties, thus, enhancing shoot proliferation. According to Uchendu and Reed (2010), shoot tips of 2 blackberry cultivars with moderate (40–60%) regrowth after standard PVS2 vitrification when treated with vitamin E (11–15 mM) had improved in vitro shoot regrowth after cryopreservation (80%) compared to the controls (50%). Sattler et al. (2004) showed that tocopherols were vital in preventing lipid peroxidation in seeds of *Arabidopsis thaliana* L. during germination. It is evident that α-tocopherol acts as an antioxidant in *S. raeseri* plants defense system under in vitro conditions and stimulates the positive effect of ZT + NAA on adventitious shoot formation pointing out a possible synergism between α-tocopherol and cytokinin + auxin combination.

Conclusions

In conclusion, 10 μM SNP or 10–50 μM $CoCl_2$, each applied separately intensified the stimulatory effect of the cytokinin BA (2.2 μM) on shoot proliferation in terms of shoot number and shoot multiplication percentage. α-Tocopherol at 11.6 μM acted synergistically with 2.2 μM ZT and 0.2 μM NAA in promoting shoot proliferation. Therefore, $CoCl_2$ or α-tocopherol to a greater extent, but SNP to a lesser one due to mild hyperhydricity symptoms that causes to explants, each substance applied separately in combination with plant growth regulators (cytokinins alone and with auxins) can potentially constitute promising shoot proliferation agents in in vitro culture of several *Sideritis* species, as it was proved for *S. raeseri* studied in this research.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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