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Effect of The Ethylene Inhibitor "Agno$_3$", Vitamin B9 "Folic Acid" And Thiol Compound "GSH" On *in Vitro* Propagation of *Sideritis Syriaca* L. Subsp. *Syriaca* (Hellenic Mountain Tea of The Crete Island)

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Abstract

The aim of this research was to study the effect of the ethylene inhibitor "silver nitrate (AgNO3)" and vitamin B9 "folic acid" in different concentrations combined with cytokinin BA as well as the antioxidant thiol compound "L-glutathione reduced (GSH)" in different concentrations simultaneously with auxins (IBA+NAA) on micropropagation efficiency of the endemic *Sideritis syriaca* L. subsp. *syriaca* (Hellenic mountain tea of Crete/ Malotira) using shoot tip explants. The culture medium used was the MS supplemented with 30 g/l sucrose. The simultaneous application 5 μM $AgNO_3$ + 2.2 μM BA promoted best the initial shoot induction stage exhibiting 4.5 shoots/ explant and 100% shoot multiplication (5 weeks). Folic acid applied at 0.25 mg/l in combination with 0.5 mg/l BA exhibited the highest shoot multiplication percentage (90%) (4 weeks). GSH at 10 μM with 10.7 μM NAA + 4.92 μM IBA gave the greatest root length (13.68 mm), at 25 μM caused a 3-fold increase in rooting (90%) and 250 μM GSH raised by 20% shoot multiplication (80%). An 89% final survival rate of rooted microplants to *ex vitro* unheated greenhouse conditions was recorded within 8 weeks period during mid-late spring. Thus, the acclimatization and hardening process was successfully completed.

Indexing Terms/Keywords: Ethylene Inhibitors, Folic Acid, Glutathione, Greek Flora, Micropropagation, *Sideritis syriaca*, Silver Nitrate, Thiol Compounds, Vitamins

Introduction

Many species of the Lamiaceae family are considered to be of high importance because of their uses in medicine, cooking, and cosmetics. Recent reports show that the main constituents of the genus *Sideritis* are various terpenoids, sterols, coumarins, and flavonoid aglycones and glycosides [1]. Many *Sideritis* species, their extracts, and constituents have also been reported to have different biological properties, such as anti-inflammatory [2, 3] and antioxidant activities [4]. According to Dimopoulos et al. [5, 6], *S. syriaca* L. subsp. *syriaca* is a native Greek endemic chamaephyte that thrives in habitats with high mountain vegetation and is distributed in Crete and Karpathos islands.

The involvement of ethylene in plant tissue growth and differentiation has been widely investigated. Application of ethylene precursors and inhibitors has shown that ethylene may often have diverse effects in similar tissue culture systems. Although it has been reported that ethylene may promote callus growth [7], it generally appears to inhibit shoot regeneration [8]. Silver nitrate ($AgNO_3$), a potent inhibitor of ethylene action [9], was shown to promote regeneration in *Brassica campestris* [10] and *Helianthus annuus* [11]. $AgNO_3$ antagonistic action on ethylene and influence on direct organogenesis had been well documented previously. $AgNO_3$ is known to enhance shoot proliferation in *Caffea canephora* [12], *Vitex nedungo* [13], *Momordica cymbalaria* [14], gloxina (*Sinnigia speciosa*) [15], shoot organogenesis [16], microspore embryogenesis [17] and *in vitro* flowering [18].

Effect of nitrate supplementation in media has been well established in tissue culture [19] so as to enhance shoot multiplication and somatic embryogenesis. Several researchers assume that NO^{-3}/NH^{4+} ratio *in vitro* acts as buffering stabilization of medium pH resulting in the organization by adventitious shoots (apical meristems) and

NO_3 subsequently promotes extension growth of these meristems into full-fledged shoots. $AgNO_3$ works as an inhibitor of ethylene activity through the Ag^{2+} ions by reducing the receptor capacity to bind ethylene [20]. With these observations, water solubility, and lack of phytotoxicity at effective concentration led to its application in tissue culture [9].

Ethylene inhibitors are added to plant media for enhancing shoot regeneration and preventing the negative effects of the ethylene hormone [15, 21]. In some species, $AgNO_3$ improved callus proliferation [22] and promoted root formation [23]. In other species, $AgNO_3$ inhibited shoot regeneration [24]. These results indicate that the promotive function of $AgNO_3$ on shoot regeneration is species-specific. Silver ions in the form of nitrate, such as $AgNO_3$, play a major role in influencing somatic embryogenesis, shoot formation and efficient root formation, which are the prerequisites for successful genetic transformation [25-27]. Addition of $AgNO_3$ to culture media greatly improved the regeneration of both dicot and monocot plant tissue cultures [28].

Vitamins, in combination with other media constituents, have been shown to have direct and indirect effects on callus growth, somatic growth, rooting, and embryonic development [29]. Vitamins such as biotin, folic acid, ascorbic acid, pantothenic acid, tocopherol (vitamin E), riboflavin, p-aminobenzoic acid are used in some cell culture media. However, they are not growth limiting factors [30]. Folic acid (vitamin B9) has become the most prominent of B complex vitamins despite its essential biochemical function in amino acids metabolism and nucleic acids synthesis [31]. Recently, great attention has been focused on the possibility of using natural and safety substances to improve plant growth. In this concern, vitamins have synergistic effects on growth, yield, and yield quality of many plant species. These compounds have beneficial effects on catching the free radicals or the active oxygen that producing during photosynthesis and respiration processes [32]. Leaving these free radicals without chelating or catching leads to lipids oxidation and the loss of plasma membrane permeability and the death of cell within plant tissues [33]. Vitamins also have an auxinic action. One of the most familiar vitamins is ascorbic acid, vitamins B, and folic acid, which are being synthesized in higher plants and affect plant growth and development [34]. Dat et al. [35] stated that the primary role of vitamins is to prevent degradation induced by free radical reactions. The presence of folic acid and biotin in the olive substrate plays an important role in the transfer of cytokinins [36]. Scott et al. [37] reported that folic acid coenzymes are involved in carbon atom transport reactions such as those necessary for the biosynthesis of methionine, serine, deoxythymic acid and purines, substances necessary for cell differentiation. The addition of some vitamins, sources of vitamin B, such as biotin, folic acid, and yeast extract, plays an important role in plant growth and the development of metabolism [38]. In tissue culture *in vitro*, some plants are deficient in vitamin synthesis [39]. Folic acid is essential for cells to replicate their DNA during cytotoxicity, as well as for RNA synthesis or DNA code reading [36].

Low molecular weight antioxidants, such as ascorbate, glutathione, and tocopherol, are information-rich redox buffers that interact with numerous cellular components. In addition to crucial roles in defense and as enzyme cofactors, cellular antioxidants influence plant growth and development by modulating processes from mitosis and cell elongation to senescence and death [40]. The localized activity of glutathione could also help elucidate the mechanism of stress resistance. This effect indicates that glutathione may be involved in protection against DNA damage [41]. Glutathione is a small, ubiquitous molecule that is involved in a plethora of cellular processes in addition to its role as an antioxidant and the maintenance of cellular redox homeostasis [42]. Glutathione is crucial for biotic and abiotic stress management, as is a pivotal component of the glutathione-ascorbate cycle, a system that reduces poisonous hydrogen peroxide [43]. It is the precursor of phytochelatins, side-chain, and an antioxidant, preventing damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides [44]. Glutathione is found almost exclusively in its reduced form, since the enzyme reverting it from its oxidized form, glutathione reductase, is constitutively active and inducible upon oxidative stress. The ratio of reduced glutathione to oxidized glutathione [45] within cells is often used scientifically as a measure of cellular toxicity. Some scientists suggest that rooting of micropropagated plants can be improved by treatment with antioxidants [46]. Antioxidants can potentially protect the natural plant rooting hormones from oxidation, enhancing rooting, and increasing the tolerance of plants to greenhouse conditions [47].

The aim of the present study concerns a contribution to the development of an efficient *in vitro* propagation procedure by assessing the influence of N6-benzyl adenine (BA) incorporation with $AgNO_3$, an ethylene action inhibitor, to assess whether it promotes or not shoot formation in *S. syriaca* subsp. *syriaca*. Furthermore, the exogenous application of vitamin B9 "folic acid" combined with cytokinin BA was tested to find out the optimum concentration of folic acid enhancing the *in vitro* shoot multiplication stage. Another aim was to study the influence of the thiol compound GSH in a wide range of concentrations to induce shoot proliferation and rooting of Crete mountain tea shoot-tip explants in combination with 2 different auxins (NAA and IBA) so as to verify if and up to what extent the combined effect of GSH+NAA+IBA exhibits better results than the individual application of auxins combination NAA+IBA under *in vitro* conditions.

Materials and methods

Experiment 1: Effect of $AgNO_3$ on *in vitro* shoot proliferation of *S. syriaca* subsp. *syriaca*

Plant material and culture conditions

The experimental plant material used was shoot-tip explants 1-2 cm long derived from previous *in vitro* cultures maintained on a Murashige and Skoog (MS) nutrient culture medium [48] in the absence of plant growth regulators (PGRs). The basal culture medium used for the experimental procedure was the MS, including all the necessary macroelements, microelements, vitamins, and amino acids. The ethylene inhibitor $AgNO_3$ (Sigma-Aldrich) was incorporated into the MS culture medium at 8 increasing concentrations (0, 1, 2.5, 5, 10, 25, 50, 100 μM) in combination with 2.2 μM BA (Sigma-Aldrich). The MS medium was enriched with 30 g/l sucrose (Duchefa) as a carbon source and 3 g/l Phytagel (Duchefa) as a gelling agent. The pH of the media was adjusted to 5.8 before adding the coagulant. After that, the coagulant was added to the conical flasks to solidify the substrate, followed by heating under continuous stirring until the solutions became clear. Subsequently, the culture media were distributed in 25 mm (width) x 100 mm (height) flat-base glass test tubes. The sterilization of the culture media was done in a liquid sterilizing autoclave at 121 °C for 20 min. After sterilization, the culture media within test tubes were cured at room temperature and used immediately within a few days to introduce the plant material (one explant transferred in each tube). The experiment included 8 treatments with 10 replicates/ treatment. After 5 weeks of culture, the percentages of shoot multiplication and vitrification were recorded, and observations were made regarding the number of shoots/ explant produced and the average length of the multiple shoots (mm) for each treatment.

Experiment 2: Effect of folic acid on *in vitro* shoot proliferation of *S. syriaca* subsp. *syriaca*

Plant material and culture conditions

Shoot tip explants 1.5-2 cm long from previous *in vitro* cultures maintained on an MS nutrient culture medium [48] were used as experimental starting plant material in the absence of plant growth regulators (PGRs). The culture medium used for the experiment was the MS including all the necessary macroelements, microelements, vitamins, and amino acids, 30 g/l sucrose (Duchefa), and 0.5 mg/l 6-benzyladenine (BA, Sigma- Aldrich). The pH of the solution was adjusted to 5.8. Vitamin B9 "folic acid" (Sigma-Aldrich) was incorporated into the MS culture medium at six increasing concentrations (0, 0.25, 0.5, 1, 2, 4 mg/l). Phytagel (Duchefa) at a concentration of 3 g/l was used as a gelling agent. Explants were placed on glass tubes with a flat base (dimensions: 25mm width x 100 mm diameter), one explant/tube. The sterilization of the culture medium was done in an autoclave at 121°C for 20 min. The experiment included six treatments with 10 replicates/ treatment. After four weeks, shoot multiplication, callusing, and vitrification percentages (%) were recorded, and observations were taken regarding the number of shoots/ explant and the average length of shoots (mm) for each treatment.

Experiment 3: Effect of GSH on micropropagation of *S. syriaca* subsp. *syriaca*

Plant material and culture conditions

The experimental plant material used was shoot-tip explants 2.5-3.5 cm long derived from previous *in vitro* cultures maintained on an MS nutrient culture medium [48] in the absence of plant growth regulators (PGRs). The basal culture medium used for the experimental procedure was the half strength (½) MS including 50% macroelements, 50% microelements, 100% vitamins, and amino acids. The thiol compound GSH (Sigma-Aldrich) was incorporated into the basal ½ MS medium at nine increasing concentrations (0, 2.5, 5, 10, 25, 50, 100, 250, 500 µM) in combination with 10.74 µM NAA and 4.92 µM IBA (Sigma-Aldrich). The MS medium was enriched with 30 g/l sucrose (Duchefa) as a carbon source and 3 g/l Gelrite (Duchefa) as a gelling agent. The pH of the media was adjusted to 5.8 prior to adding the coagulant. After that, the coagulant was added to the conical flasks to solidify the substrate, followed by heating under continuous stirring until the solutions became clear. Subsequently, the culture media were distributed in 25 mm (width) x 100 mm (height) flat-base glass test tubes. The sterilization of the culture media was done in a liquid sterilizing autoclave at 121 °C for 20 min. After sterilization, the culture media within test tubes were cured at room temperature and used immediately within a few days to introduce the plant material (one explant transferred in each tube). The experiment included 9 treatments with 10 replicates/ treatment. After 8 weeks of culture, the percentages of shoot multiplication, vitrification, rooting and callusing were recorded, and observations were made regarding the number of shoots / explant, the average length of the multiple shoots (mm), root number/ rooted micro-cutting and root length (mm).

***Ex Vitro* acclimatization of rooted *S. syriaca* subsp. *syriaca* microplantes**

With respect to the acclimatization process, the well-rooted shoots derived from the culture medium containing different GSH concentrations (Experiment No. 3) were removed from the culture tubes and washed thoroughly to remove the traces of agar. In mid-April, the plantlets of *in vitro* grown *S. syriaca* subsp. *syriaca* with well-developed roots and shoots were transplanted to 0.25 Lt pots containing an enriched peat moss (Terrahum): perlite (Perflor) substrate (1:1) and transferred to the mist system under unheated greenhouse conditions. After a 4-week period (mid-May) of maintenance in the mist, the survival rate of the successfully acclimatized plantlets was recorded. Following another 4-weeks interval (mid-June), the final survival rate was noted and the hardened plants were subsequently transferred to larger pots (1 Lt) containing a peat moss more enriched in organic matter (TS2, Klasmann), perlite and soil (2:1:1) substrate and were allowed to grow under nursery shade conditions, in external environmental conditions. In the outside nursery, the plants were watered 2 times/ day by sprinkling and were finally planted in field conditions.

Statistical analysis

The statistical analysis of all three individual fully randomized experiments was performed using the ANOVA (Analysis of Variance) and SPSS 17.0 (SPSS Inc., Illinois, New York, USA) for a 5% significance level based on the Duncan criterion. In tables, the numbers with different letters ± standard error (S.E.) in each column and for each macroscopic characteristic separately differ considerably from one another to a 5% significant level ($p \leq 0.05$).

Results

Experiment 1: Effect of $AgNO_3$ on *in vitro* shoot proliferation of *S. syriaca* subsp. *syriaca*

$AgNO_3$, when added to the MS medium in the 5-100 µM concentration range positively influenced the number of shoots (4-4.6 shoots/ explant) in relation to the control (2.56 shoots/ explant). The lowest applied $AgNO_3$ concentration of 1 µM significantly increased the length of the shoots by 0.5 cm (from 15.42 to 20.44 mm) while higher $AgNO_3$ concentrations (2.5-100 µM) did not cause a substantial change in the elongation of the produced multiple shoots. All $AgNO_3$ treatments (1-100 µM) enhanced the ability of explants to induce multiple shoots (80-100%) compared to the control (77.78%). The shoot multiplication percentage was 100% when the explants treated with 5-25 µM $AgNO_3$. The number of shoots was maximum (5.22) under the effect of 100 µM $AgNO_3$

and the length of shoots greater when 1 µM $AgNO_3$ was incorporated into the medium. Hyperhydricity problems were observed to the explants in all treatments with $AgNO_3$, including the control's ($AgNO_3$-free) with a vitrification percentage ranging between 30% and 55.56% (Table 1, Fig. 1A-1H).

Table 1. Effect of $AgNO_3$ concentration (0-100 µM) combined with 2.2 µM BA on shoot number/ explant, shoot length (mm), shoot multiplication and vitrification percentages (%) in *S. syriaca* after 5 weeks of culture (MS medium supplemented with 30 g/l sucrose and 3 g/l Phytagel)

$AgNO_3$ (µM)	Shoot number/ explant	Shoot length (mm)	Shoot multiplication (%)	Vitrification (%)
0	2.56 ± 0.34 a	15.42 ± 1.72 ab	77.78 a	44.44 d
1	3.89 ± 0.60 abc	20.44 ± 5.06 b	88.89 c	44.44 d
2.5	3.30 ± 0.63 ab	12.52 ± 1.52 a	90 c	30 a
5	4.50 ± 0.30 bc	12.14 ± 0.89 a	100 d	37.50 b
10	4.00 ± 0.60 abc	15.45 ± 1.10 ab	100 d	55.56 e
25	4.30 ± 0.52 bc	13.04 ± 0.92 a	100 d	40 c
50	4.60 ± 0.53 bc	13.50 ± 0.31 a	80 b	40 c
100	5.22 ± 0.53 c	13.50 ± 1.36 a	88.89 c	44.44 d
P-values	0.022*	0.142 ns	0.000***	0.000***

Means ± S.E. with the same letter in a column is not statistically significantly different from each other, according to Duncan's multiple range test at P ≤ 0.05. ns P ≥ 0.05, *P ≤ 0.05, ***P ≤ 0.001.

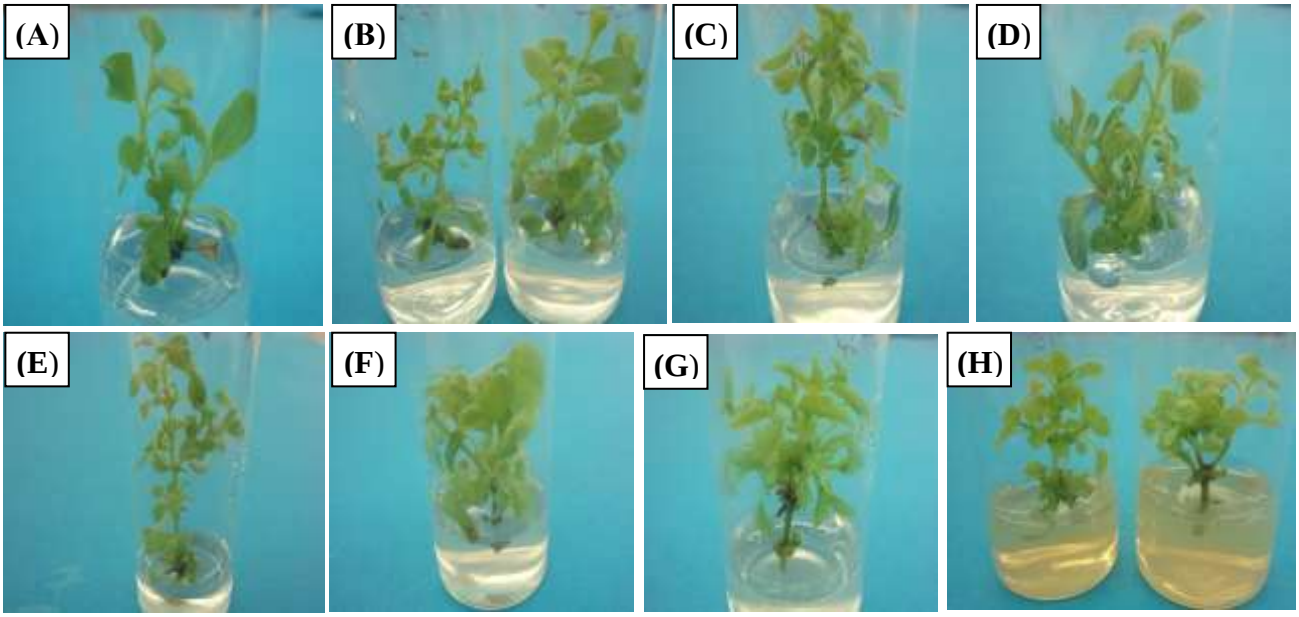

Figure 1. Effect of the ethylene inhibitor "$AgNO_3$" concentration on *in vitro* shoot multiplication of *S. syriaca* subsp. *syriaca* explants **(A)** Control ($AgNO_3$-free), **(B)** 1 µM, **(C)** 2.5 µM, **(D)** 5 µM, **(E)** 10 µM, **(F)** 25 µM, **(G)** 50 µM and **(H)** 100 µM $AgNO_3$

Experiment 2: Effect of folic acid on *in vitro* shoot proliferation of *S. syriaca* subsp. *syriaca*

Folic acid (0.25-4 mg/l) did not substantially alter the number of shoots (2.4-3.6 shoots/explant) and the average shoot length (14.69-17.96 mm) compared to the control (2.2 shoots/ explant 17.14 mm long), however significantly promoted shoot proliferation increasing the percentage of explants with multiple shoot production (70-90%) with respect to the control (50%). In specific, shoot multiplication percentage was higher (90%) under the influence of the two extreme concentrations of folic acid, 0.25 and 4 mg/l, respectively, while at the intermediate concentrations (0.5-2 mg/l) ranged between 70% and 80%. In the control treatment, no callus formation was observed at the base of the explants, while adding folic acid (0.25-4 mg/l) resulted in callus induction to the 20-70% of the explants. Hyperhydricity symptoms were evident in all treatments, including the control. The two lower folic acid concentrations (0.25, 0.5 mg/l) gave 40% vitrification percentage, while higher concentrations (1-4 mg/l) increased the incidence of this physiological phenomenon (60-70%). Callusing and vitrification percentages were higher (70%) when explants treated with 2 mg/l folic acid (Table 2, Fig. 2A-2F).

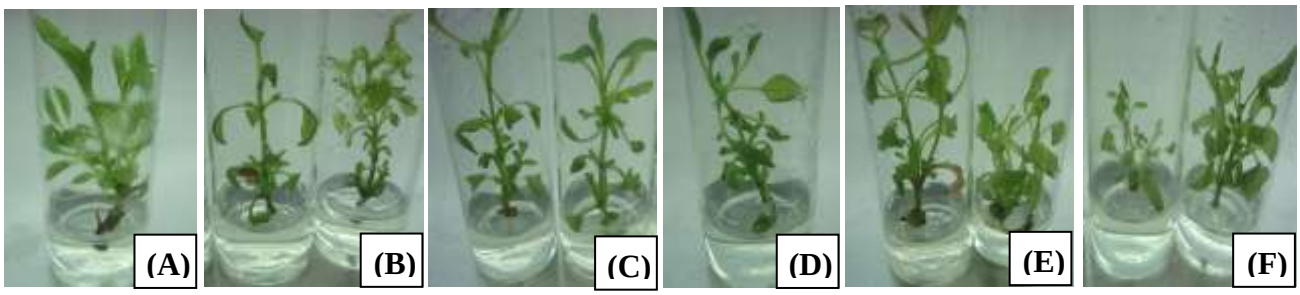

Figure 2. Effect of folic acid (vitamin B9) concentration on *in vitro* shoot multiplication of *S. syriaca* subsp. *syriaca* expants **(A)** Control (folic acid-free), **(B)** 0.25 mg/l, **(C)** 0.5 mg/l, **(D)** 1 mg/l and **(E)** 2 mg/l folic acid

Table 2. Effect of folic acid concentration (0-4 mg/l) combined with 0.5 mg/l BA on average shoot number/ explant, shoot length (mm), shoot multiplication, callusing and vitrification percentages (%) in *S. syriaca* after 4 weeks of culture [MS medium supplemented with 30 g/l sucrose and 3 g/l Phytagel]

Folic acid (mg/l)	Shoot number/ explant	Shoot length (mm)	Shoot multiplication (%)	Callusing (%)	Vitrification (%)
0	2.20 ± 0.47 a	17.14 ± 2.26 a	50 a	0 a	40 a
0.25	3.50 ± 0.52 a	16.13 ± 0.74 a	90 d	20 a	40 a
0.5	2.90 ± 0.46 a	17.96 ± 1.25 a	70 b	40 c	40 a
1	2.67 ± 0.39 a	17.64 ± 1.51 a	77.77 c	66.67 de	66.67 c
2	2.40 ± 0.31 a	20.58 ± 3.89 a	80 c	70 e	70 d
4	3.60 ± 0.56 a	14.69 ± 1.15 a	90 d	60 d	60 b
P-values	0.188 ns	0.486 ns	0.000***	0.000***	0.000***

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

Experiment 3: Effect of GSH on micropropagation of *S. syriaca* subsp. *syriaca*

GSH (2.5-500 µM) did not differentiate shoot number (1.6-3.4 shoots/ explant) and shoot length (15.5-21.55 mm) with respect to the control (2.2 shoots/ explant 17.88 mm long). Shoot multiplication percentage was highest (80%) and increased by 20% compared with the control when explants treated with 250 µM GSH. The 50 or 100 µM GSH + 10.74 µM NAA + 4.72 µM IBA combination treatments eliminated hyperhydricity symptoms

(0% vitrification), while 250 M GSH gave the same vitrification percentage to the control (20%). No symptoms of necrosis were evident in control GSH-untreated explants. However, GSH regardless of concentration applied, led to the appearance of necrotic/browning symptoms. In particular, necrosis symptoms percentage was only 10% when the medium was fortified with low (2.5 µM) and high (100-500 µM) GSH concentrations. Taking into consideration all the macroscopic parameters, 250 µM GSH exhibited better shoot proliferation results (Table 3, Fig. 3A-3I).

In all treatments, a simultaneous shoot proliferation and rooting were observed as a result of GSH exogenous application. GSH did not raise further root number (4.5-10 roots/ rooted micro-cutting) about the control (8.67 roots/ rooted micro-cutting). Root length was increased by 0.3 cm, from 10.94 mm (control) to 13.68 mm with 10 µM GSH. All GSH concentrations promoted rooting by raising the respective percentage from 30% (control) to 40-90%. The percentage of explants with root formation was higher (80%, 90%, and 70%) under the effect of 2.5 µM, 25 µM and 100 µM GSH, respectively by exhibiting at the same time 30%, 80%, and 0% vitrification, accordingly, therefore, 100 µM GSH was the best option for the *in vitro* rooting stage. Callus formation occurred in almost all GSH treatments including the control to the 10-80% of the explants, except for 50 and 100 µM concentrations, in which no callusing was observed (Table 4, Fig. 3A-3I).

Table 3. Effect of GSH concentration (0-500 µM) combined with 10.7 µM NAA and 4.92 µM IBA on average shoot number/ explant, shoot length (mm), shoot multiplication, vitrification and necrosis percentages (%) in *S. syriaca* after 8 weeks of culture [½ MS medium (50% macro-, 50% micronutrients, full-strength vitamins) supplemented with 30 g/l sucrose and 3 g/l Gelrite)

GSH (µM)	Shoot number/ explant	Shoot length (mm)	Shoot multiplication (%)	Vitrification (%)	Necrosis (%)
0	2.20 ± 0.36 a	17.88 ± 1.37 a	60 d	20 c	0 a
2.5	2.30 ± 0.37 a	16.63 ± 1.36 a	70 e	30 d	10 b
5	1.60 ± 0.31 a	17.75 ± 1.80 a	40 b	10 b	40 d
10	2.30 ± 0.78 a	19.25 ± 1.40 a	50 c	40 e	20 c
25	2.40 ± 0.52 a	18.90 ± 3.65 a	50 c	80 f	50 e
50	1.80 ± 0.51 a	21.33 ± 2.25 a	30 a	0 a	40 d
100	3.30 ± 0.96 a	21.55 ± 2.50 a	60 d	0 a	10 b
250	3.40 ± 0.58 a	15.50 ± 2.81 a	80 f	20 c	10 b
500	2.00 ± 0.49 a	18.42 ± 2.68 a	50 c	10 b	10 b
P-values	0.347 ns	0.666 ns	0.000***	0.000***	0.000***

Means ± S.E. with the same letter in a column is not statistically significantly different from each other according to the Duncan's multiple range test at P ≤ 0.05. ns P ≥ 0.05, ***P ≤ 0.001.

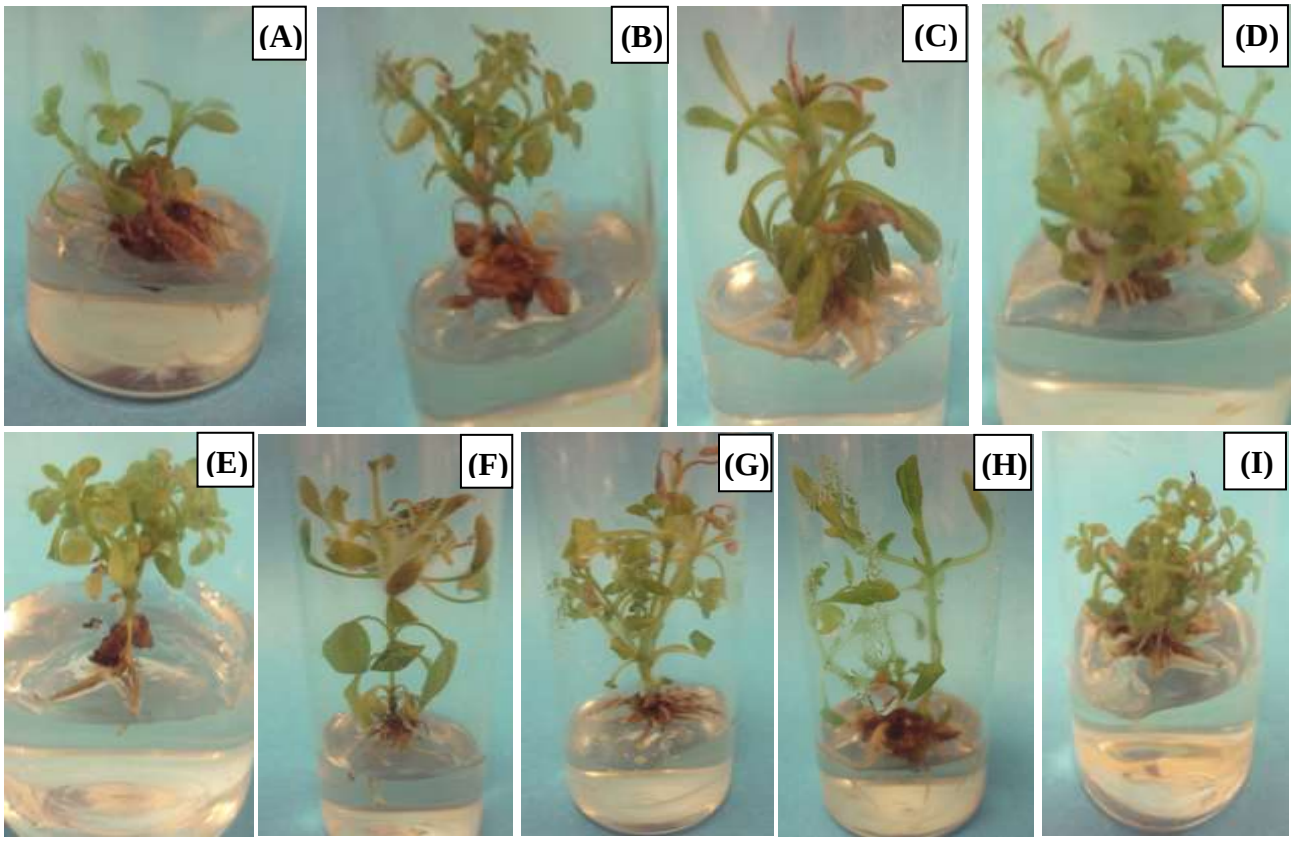

Figure 3. Effect of the antioxidant thiol compound "GSH" concentration on *in vitro* shoot multiplication and rooting of *S. syriaca* subsp. *syriaca* expants **(A)** Control (SGH-free), **(B)** 2.5 µM, **(C)** 5 µM, **(D)** 10 µM, **(E)** 25 µM, **(F)** 50 µM, **(G)** 100 µM, **(H)** 250 µM and **(I)** 500 M GSH

Table 4. Effect of GSH concentration (0-500 µM) combined with 10.7 µM NAA and 4.92 µM IBA on root number/ rooted micro-cutting, root length (mm), rooting and callusing percentages (%) in *S. syriaca* after 8 weeks of culture [½ MS medium (50% macro-, 50% micronutrients, full-strength vitamins) supplemented with 30 g/l sucrose and 3 g/l Gelrite)

GSH (µM)	Root number	Root length (mm)	Rooting (%)	Callusing (%)
0	8.67 ± 0.48 cd	10.94 ± 0.53 d	30 a	20 c
2.5	6.38 ± 0.93 abc	8.41 ± 0.54 abc	80 e	30 d
5	5.00 ± 0.98 ab	8.51 ± 0.52 abc	40 b	10 b
10	4.50 ± 0.68 a	13.68 ± 1.73 e	60 c	40 e
25	9.11 ± 1.00 cd	10.42 ± 0.93 cd	90 f	80 f
50	7.50 ± 1.47 bcd	6.35 ± 0.17 a	40 b	0 a
100	8.71 ± 1.26 cd	9.08 ± 0.44 bcd	70 d	0 a
250	10.00 ± 0.75 d	9.67 ± 0.48 bcd	40 b	20 c
500	7.25 ± 0.58 abcd	7.75 ± 0.30 ab	40 b	10 b
P-values	0.001**	0.000***	0.000***	0.000***

Means ± S.E. with the same letter in a column is not statistically significantly different from each other according to the Duncan's multiple range test at P ≤ 0.05. **P ≤ 0.01, ***P ≤ 0.001.

Ex Vitro acclimatization of rooted *S. syriaca* subsp. *syriaca* microplants

About 82% of the *in vitro* rooted transplanted plantlets survived after acclimatization *ex Vitro* and showed healthy growth without any morphological variations after 4 weeks (mid-May) of maintenance in the mist of the unheated greenhouse. Finally after another 4-weeks interval (mid-June) in one of the benches of the greenhouse (outside of the mist), the hardened plants (96% survival rate) were transferred to larger pots (1 Lt) containing a peat moss more enriched in organic matter, perlite and soil (2:1:1) substrate and were allowed to grow under nursery shade conditions (Fig. 4A-4B).

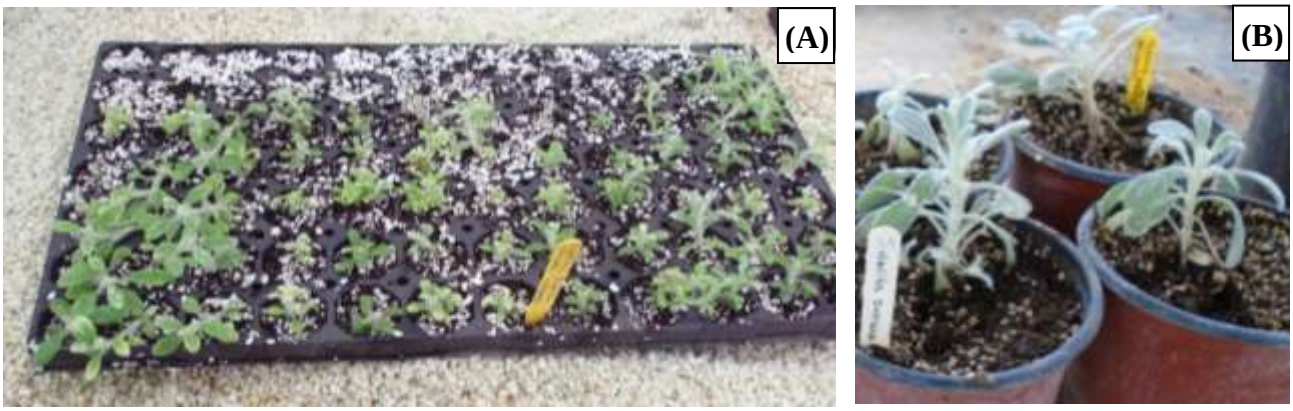

Figure 4. *Ex Vitro* acclimatization and adaptation of *in vitro* rooted *S. syriaca* subsp. *syriaca* plantlets: **(A)** internal mist system inside greenhouse conditions, planted to multi-seat discs with peat: perlite (1:1 v/v) soil substrate and watered by sprinkling for the first 4 weeks, **(B)** transplantation of plants after 4 weeks in pots of 1 Lt with peat: perlite: sand (2:1:1 v/v) soil substrate, maintenance in the greenhouse bench and watered by sprinkling

Discussion

Experiment 1: Effect of $AgNO_3$ on *in vitro* shoot proliferation of *S. syriaca* subsp. *syriaca*

Cytokinins such as BA promote the growth of axillary buds by reducing the apical dominance of buds during the micropropagation phase [49]. $AgNO_3$ is reported to be one of the potent inhibitors of ethylene. It inhibits ethylene activity through Ag ions by reducing the receptor capacity to bind ethylene [50]. $AgNO_3$ has several useful properties such as promoting root induction in *Decalepsis hamiltonii* plants [51], maturation of somatic embryos of an important medicinal plant *Andrographis paniculata* [52] and enhancement of shoot proliferation in the nodal culture of *Vanilla planifolia* [53].

In the present research study, the combined effect of the inhibitor of the ethylene action "$AgNO_3$ (1-100 µM)" with cytokinin BA (2.2 µM) promoted shoot proliferation of *S. syriaca* subsp. *syriaca* micro cuttings by acting synergistically and exhibiting better results compared to the individual effect of BA. According to a previous study conducted by Sarropoulou et al. [54] in 2 cherry rootstocks; CAB-6P (*Prunus cerasus* L.) and Gisela 6 (*Prunus cerasus* x *Prunus canescens*), $AgNO_3$ (10-50 µM) promoted proliferation concerning shoot number and shoot multiplication percentage. Similarly, $AgNO_3$ enhanced shoot regeneration of white marigold [55], cassava [56], *Vanilla planifolia* [53], *Capsicum* spp. [57], *Coffea canephora* [58], pomegranate (*Punica granatum* L.) [59], *Morinda reticulata* Gamble [60], perennial alfalfa (*Medicago sativa*) [61] and Virginia-type peanut plants [62].

Shoot number of studied *S. syriaca* subsp. *syriaca* was substantially enhanced (2-fold increase) due to $AgNO_3$ (5, 25, 50, 100 µM) application; however, shoot elongation was hardly affected by $AgNO_3$ (1-100 µM). Our findings are partly in agreement with those recorded in both Gisela 6 and CAB-6P micro-cuttings, in which shoot number was maximum with 20 µM and 30 µM $AgNO_3$, respectively [54]. Concerning shoot elongation of the 2 cherry rootstocks, 50 µM $AgNO_3$ was optimum for CAB-6P whereas in Gisela 6 except 20 µM $AgNO_3$, the remaining applied concentrations (10, 30-50 µM) resulted in a significant decrease of shoot length [54]. A similar

type of enhancement up of shoot number to two folds in gloxiana [15] (Park et al., 2012), three folds in momordica [14] and cucumis [16] had been reported earlier in the presence of $AgNO_3$ in the MS culture medium.

In the current study employing *S. syriaca* subsp. *syriaca*, $AgNO_3$ irrespective of concentration exerted a stimulating effect on explants' ability to induce multiple shoots. Similar results were obtained in CAB-6P micro cuttings where 20 μM $AgNO_3$ gave the highest shoot multiplication percentage and 30 or 40 μM $AgNO_3$ in Gisela 6 [54]. Some authors proved that $AgNO_3$ significantly increased the percentage of explants producing multiple shoots such as in *Swainsona salsula* Taubert plants [63]. The positive effect of $AgNO_3$ on shoot regeneration has already been reported for a number of plants, including cotton [64] and sesame [65]. In other plant species, shoot proliferation performance of micro-cuttings is $AgNO_3$ concentration-depended. In specific, Chae and Park [66] found that $AgNO_3$ (1–10 mg/l) increased shoot regeneration percentage, a number of shoots per explant, and shoot length in *Echinacea angustifolia*, while 20 mg/l $AgNO_3$ had a negative effect. Similar $AgNO_3$ concentration-depended response of microcuttings to shoot proliferation attributes was performed in pomegranate (*Punica granatum* L.) [67].

Taking simultaneously into consideration all 4 macroscopic characteristics (shoot number and length, shoot multiplication and vitrification percentages), 5 μM $AgNO_3$ provided optimum *in vitro* results for the stage of shoot proliferation of *S. syriaca* subsp. *syriaca* explants within a 5-week period of culture. Our findings are in consistency with those presented by Sarropoulou et al. [54] where 20 μM $AgNO_3$ in CAB-6P and 30 μM $AgNO_3$ in Gisela 6 cherry rootstocks proved to be optimal for *in vitro* shoot proliferation. Determination of optimal concentrations of $AgNO_3$ for use in micropropagation of several plant species has been reported. For instance, $AgNO_3$ at 20 mg/l was found effective in the nodal culture of *Theobroma cocoa* [68], *Coffea arabica* [69], and *Vanilla planifolia* [70].

From the results, it is clearly emphasized that the presence of an optimal concentration of $AgNO_3$ had increased shoot proliferation in *S. syriaca*. Supplementation of $AgNO_3$ in the media further significantly enhanced the high frequency of shoot regeneration. The exact mechanism of $AgNO_3$ amelioration in shoot proliferation is still unclear. However, few existing evidences suggest that silver ions are thought to perturb the ethylene ion binding site [71] and sometimes over-accumulation of ethylene in culture tubes is auto inhibitory for further production of ethylene itself [72]. Antagonistic action of $AgNO_3$ may be attributed to the gradual replacement of Cu^+ cofactors with Ag^+ ions in ethylene binding site ETR1 brings about conformational changes [73]. Rarely $AgNO_3$ may cause ethylene insensitive mutations [74]. Finally, we can also attribute the $AgNO_3$ positive effect of regeneration due to enhanced polyamine synthesis rather than reduced ethylene production [75]. Poor regeneration frequency and shoot induction response of explants, those cultured on control medium un-supplemented with $AgNO_3$ may be attributed to suppression of growth by the accumulation of ethylene in the culture tubes. This is in agreement with published reports [76].

Experiment 2: Effect of folic acid on *in vitro* shoot proliferation of *S. syriaca* subsp. *syriaca*

In the studied *S. syriaca*, taking into account all the individual parameters, namely the number of shoots/ explant (3.5), the length of the shoots (16.13 mm), shoot multiplication (90%) and vitrification (40%) percentages is recommended to incorporate 0.25 mg/l of folic acid into the MS culture medium for shoot proliferation of explants. Similar findings were obtained in three different varieties of tomato (*Lycopersicon esculentum* Mill.), Pant 11, Pant 5 & Le 79, where 0.25-1 mg/l folic acid significantly promoted *in vitro* shoot proliferation by increasing the number of shoots/ explant, shoot length and the percentage of explants with multiple shoot production [77]. In the current study, the increase in folic acid concentration did not influence the number and length of *S. syriaca* subsp. *syriaca* micro shoots. Different results related to the negative effect of folic acid on plant height were reported in other species including wheat (*Triticum aestivum L.*) [78], flax (*Linum usitatissimum*) [79] and pea (*Pisum sativum*) [80]. In this study with the Crete mountain tea, the supplementation of culture medium with folic acid led to callus induction, with the respective percentage being higher with 1-2 mg/l folic acid. The outcome of the present study concerning callusing percentage is in agreement with that achieved in the palm *in vitro* culture (*Phoenix dactylifera* L. cv. Ghazal), where the callus formation percentage was higher

when the explants were treated with 0.1 mg/l folic acid [81]. Therefore, the 0.25 mg/l folic acid + 0.5 mg/l BA combination treatment proved to be optimum for *in vitro* shoot proliferation of *S. syriaca* subsp. *syriaca* explants, taking concurrently into account both callusing and vitrification percentages.

Experiment 3: Effect of GSH on micropropagation of *S. syriaca* subsp. *syriaca*

In the studied *S. syriaca*, GSH, irrespective of concentration did not enhance further the length of the produced multiple shoots in relation to the control. However, in the apple rootstock MM106 (*Malus domestica* Borkh.), the incorporation of 10 μM GSH into the MS culture medium containing 5.4 μM NAA resulted in the greatest shoot height (41.43 mm) after 28 weeks of culture, compared with the control GSH-untreated explants [82]. Nomura et al. [83] found that 100 μM GSH improved the development of isolated shoot tips of apple. Similarly, in soybean, 100 μM GSH increased shoot length both in the presence and absence of IAA shock alone [84]. Positive effects of GSH on shoot length were also reported for green onion (*Allium cepa* L., cv. Giza 6) plants [85] and *Spilanthes calva* L. *vitro* culture [86]. In the current study employing *S. syriaca* explants, GSH concentration did not have an influence on shoot number with respect to the untreated-GSH explants. On the contrary, in gladiolus, shoot organogenesis frequency, and shoot number per explant using leaf segment explants were increased with the addition of 500 μM GSH, however, higher concentrations were found to be inhibitory [87]. In *Pistacia vera* L. shoot tip culture [88], GSH reduced the total phenolic compounds, thus promoting shoot growth. Therefore, the optimum combination treatment promoting best the initial *in vitro* shoot proliferation stage of *S. syriaca* explants taking into account all the individual shoot proliferation parameters is 250 μM GSH + 10.7 μM NAA +4.92 μM IBA (80% shoot multiplication percentage, 3.4 shoots/ explant 15.5 mm long).

Root formation in shoot cuttings of soybean (*Glycine max* L. 'Williams'), mungbean (*Phaseolus aureas* Mdlbg.), English ivy (*Hedera helix* L.), and apple *(Malus* x *domestica* Borkh. 'Jork 9') was stimulated by GSH in the presence and absence of auxin (IAA: indole-3-acetic acid) shock [84]. According to Auderset et al. [84], root number was positively influenced when 100 μM GSH was used simultaneously with IAA in both soybean and mungbean (*P. aureas* Mdlbg.) cuttings. The same authors reported that in micropropagated apple (Malus x domestica Borkh. 'Jork 9') shoots derived from callus, 25-100 μM GSH augmented the percentage of rooted explants while root number was increased in the 50-75 μM GSH concentration range. In this study with Crete mountain tea (Greek flora), root length and root number was greater with 10 μM and 250 μM GSH, accordingly. Similarly, in the apple rootstock MM106 (*Malus domestica* Borkh.), the incorporation of 10 μM GSH into the MS culture medium containing 5.4 μM NAA resulted in the maximum root number (11.67) and root length (41.11 mm), however, the highest rooting percentage (50%) was achieved in the 25 μM GSH + 5.4 μM NAA combination treatment, in relation to the control [82]. The rooting percentage of *S. syriaca* shoot-tip explants was the highest (90%) under the effect of 25 μM GSH, where the highest vitrification (80%), necrosis (50%) and callusing (80%) percentages were concurrently obtained, thus this treatment was proved to be not appropriate for the *in vitro* rooting stage. Accordingly, *in vitro* rooting of sweet cherry (*Prunus avium* L.) cv. 'Kristiina (root number and rooting percentage) was significantly promoted by fortifying the MS medium containing 9.84 μM IBA with 25 μM GSH [89]. Imin et al. [90] found that both reduced (GSH) and oxidised (GSSG) form of glutathione markedly enhance the number of roots formed by callus derived from leaf explants of *Medicago truncatula* cultured on NAA-supplemented medium than on medium supplemented with NAA alone. Tyburski and Tretyn [91] demonstrated that supplementing the rooting medium with GSH (1-2.5 mM) increased the number of roots formed by tomato seedling cuttings grown on an auxin-free medium, however, the strongest stimulation of root formation occurred when plants were simultaneously treated with auxin and GSH. Standardi and Romani [92] reported inhibition of rooting in *Malus* due to GSH application at mM concentrations. Therefore, in the studied *S. syriaca*, taking all the individual rooting attributes into consideration simultaneously with vitrification and necrosis phenomena, GSH applied at 100 μM along with the 10.7 μM NAA + 4.92 μM IBA auxins combination gave the most promising results (70% rooting percentage, 8.71 roots / rooted explant, 9.08 mm average root length). Therefore, GSH can be used as a rooting promoting agent in *S. syriaca* tissue culture system.

With respect to the studied species-subspecies, the callusing percentage was higher (80%) when explants treated 25 μM GSH, no callus formation occurred with 50 and 100 M GSH while the percentage of explants with

callus formation in the remaining treatments was ranged between 10% and 40%. In the apple rootstock MM106 (*Malus domestica* Borkh.), callusing percentage (57.14%) was maximum by adding 10 µM GSH to the medium. No callusing was recorded in the absence of GSH and when explants treated with 5, 100, 250, or 1000 µM GSH [82]. In apple *vitro* culture, GSH promoted callus growth [83]. In yew (*Taxus baccata* L.), callogenesis percentage was significantly increased when 100 µM GSH were added to the culture medium [93].

The mechanism by which thiol compounds might enhance rooting is unknown. For example, GSH reduces auxin effects by forming conjugates [94]. However, there is no precedent for potentiation of an auxin response by thiols. Our findings may be of theoretical importance concerning a potential auxin x thiol interaction in plant growth and differentiation and of practical importance to artificial rooting of woody and herbaceous shoot micro-cutting.

Ex Vitro **acclimatization of rooted *S. syriaca* subsp. *syriaca* microplants**

Recent advances in plant tissue culture regarding the *ex vitro* field evaluation of acclimatized plants have resulted in the development of protocols for micropropagation of many aromatic plants. The transplantation stage continues to be a major bottleneck in the micropropagation of aromatic plants. Plantlets grown *in vitro* have been continuously exposed to a unique microenvironment and have been selected to provide minimal stress to achieve optimum conditions for rapid multiplication. Acclimatization of micro propagated plant to a greenhouse, or a field environment is essential because anatomical and physiological characteristics of *in vitro* plantlets necessitate that they should be gradually acclimatized to the environment of the greenhouse or field [95]. Successful acclimatization procedures provide optimal conditions for a high percentage of survival of plants, they minimize the percentage of dead and damaged plants in the micropropagation process, and they enhance the plant growth and establishment [96]. In the studied *S. syriaca* subsp. *syriaca*, the process of gradual acclimatization and hardening of the *in vitro* rooted plantlets to the *ex vitro* greenhouse conditions during mid-April - mid-June period was successful exhibiting 82% first survival rate under internal mist conditions and 96% final survival before their transition into natural conditions. The successfully survived *S. syriaca* subsp. *syriaca* plantlets following subsequent transplantations showed healthy growth without any morphological variations. In the current study, the whole process of acclimatization and gradual hardening of *S. syriaca* subsp. *syriaca* microplants to the *ex vitro* environment lasted almost 2 months in the mid spring-early summer season. Efficient acclimatization procedure saves the resources of time, labor, and money, reducing the cost of production of qualified and deliverable products [97]. Dynamics of the process as well as the final percentage of fully acclimatized plants are related to plant species and both *in vitro* and *ex vitro* culture conditions [98]. Some plant species are unable to adapt *in vitro* formed leaves to *ex vitro* conditions, but leaves of many other plant species such as *S. syriaca* subsp. *syriaca* are fully capable of *ex vitro* acclimatization, and they function until new leaves are formed [99].

Conclusions

The *in vitro* regeneration of some plants remains difficult due to the high degree of callusing, high phenolic excretion into the medium and consequent blackening of explants. Fortification of culture media with different PGRs, i.e. auxins and cytokinins is not enough to regenerate the plant with high efficiency. This type of cultures in some cases may be improved by incorporation of additives in the media such as $AgNO_3$ due to their growth and development promoting activities to induce *in vitro* regeneration of an important endangered medicinal species *S. syriaca* subsp. *syriaca*. Hence micropropagation can be strategically improved by modifying the hormonal composition in the media and their interaction with certain inhibitors. In our present investigation, *in vitro* propagation in the presence of $AgNO_3$ had shown marked significance in almost all parameters in comparison with media without $AgNO_3$, being optimum at 5 µM. The presence of folic acid in the culture affects plant growth and development related to shoot proliferation of *S. syriaca* subsp. *syriaca* explants, playing an important role in the transfer of cytokinins such as BA, thus exhibiting a synergistic action as better results were achieved in the 0.25 mg/l folic acid – 0.5 mg/l BA combination treatment than the individual effect of cytokinin alone. GSH in combination with auxins participates in plant regeneration of *S. syriaca* subsp. *syriaca*, promoting

rooting and shoot proliferation being optimum with 2.5 µM and 250 µM GSH accordingly. It is obvious that GSH is involved in mechanisms regulating cell divisions in newly formed meristems and participating in hormone metabolism and signaling. Diverse functions of this antioxidant open vast possibilities of using it for the improvement of tissue culture and plant regeneration methods. However, further studies are required to fully exploit the properties of GSH for manipulating developmental processes in plant tissue culture.

Conflicts of Interest

The authors declare no conflict of interest in this work

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