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Effects of two abiotic elicitors on secondary metabolites accumulation and bioactivity in tree wormwood *in vitro* shoot cultures

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ABSTRACT: *Artemisia arborescens* L., well known as tree wormwood, is a medicinal species of the Asteraceae family that makes a part of the folk medicine system in the Mediterranean regions. Several scientific studies have validated the traditional medicinal use of tree wormwood and confirmed its antimicrobial, anti-tumor, antioxidant, anti-inflammatory and hepatoprotective properties. In this study, the incidence of two abiotic elicitors (NaCl and KCl) on the concentration and bioactivity of secondary metabolites in tree wormwood microshoots regenerated *in vitro* was evaluated. The obtained results showed that the application of 100 mM NaCl or KCl to one-month old *in vitro* developed microshoots resulted in the enhancement of the accumulation of total polyphenols, flavonoids, condensed tannins and volatile compounds. The highest improvement rates of total polyphenols (80%) and flavonoids (53%) over the control were obtained with NaCl elicitor. However, KCl elicitor has been shown more efficient in the enhancement of condensed tannins (430%) and volatile metabolites (39%) contents. The application of the two salt elicitors led also to a significant increase of the antioxidant activities of ethanolic and volatile metabolites extracts in the treated microshoots. The obtained results are promising and further detailed investigations are needed for the optimization of this strategy to improve the *in vitro* production of *Artemisia arborescens* L. phytochemicals with high potentialities in alternative medicine and pharmaceutical industry.

1. INTRODUCTION

The interest given to medicinal plants and their extracts in the prevention and treatment of human diseases dates from the oldest times to the recent Chinese, Indian and Near Eastern civilizations (Giannenas et al., 2020). Even after the recorded progress in chemical drug industry, the attention attributed to herbal products is still gaining importance (Negi et al., 2018). The last decade, the commercial demands and industrial needs of medicinal plants, with increasing economic, social, medical and ecological importance, recorded a significant increase worldwide (Taghouti et al., 2022).

The health promoting effects and virtues of medicinal plants depend mainly on their richness in specific phytochemicals of the secondary metabolites group. Plant secondary metabolites form a rich class of bioactive molecules which provides a natural resource for the discovery and development of new drugs, cosmetics, agrochemicals and functional foods (Chiocchio et al., 2021). Despite their interest, plant secondary metabolites are accumulated in low amounts, generally less than 1% of plant material (Thakur et al., 2019). Based on the last considerations, the enhancement of the concentration levels of these high-value phytochemicals is the main challenge for plant biotechnologists and breeders (Lema-Rumińska et al., 2019; Riahi et al., 2020). Conventional breeding strategies exhibited limits in the improvement of secondary metabolites traits mainly due to their complex biosynthetic pathways and the significant fluctuation of their accumulation under the effect of various endogenous and exogenous factors (Sinha et al., 2019).

The production of plant secondary metabolites of interest under *in vitro* cultures systems has seen renewed interest the

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last years for aromatic and medicinal crops and provides wide range of advantages (Wawrosch & Zotchev, 2021). Indeed, *in vitro* culture technologies under controlled conditions offer the possibility of enhancing the quantity and quality of these phytochemicals by controlling the factors which affect their synthesis and accumulation (Singh et al., 2018). Thus, the application of different types of biotic and abiotic stress or signal molecules called elicitors to the *in vitro* cultures has allowed the improvement of secondary metabolites contents and sometimes the accumulation of new compounds (Thakur et al., 2019; Yazdanian et al., 2022). This approach was reported as an alternative, simple and low-cost method for the sustainable production of plant secondary metabolites with commercial and industrial demands (Halder et al., 2019).

The *in vitro* application of various types of elicitors including abiotic stress, nanoparticles, jasmonic acid, salicylic acid among others has been shown efficient to enhance the production of useful bioactive compounds belonging to the classes of terpenoids, phenolics, flavonoids, alkaloids in various plant species (Ayoola-Oresanya et al., 2021; Khan et al., 2021; Yazdanian et al., 2022). In this study, the effect of NaCl and KCl used as two abiotic elicitors on the concentration of phenolics and volatile metabolites in the *in vitro* regenerated microshoots of *Artemisia arborescens* L. was investigated. The main objectives are to evaluate the incidence of these two elicitors supplemented in the culture medium at a concentration of 100 mM on the accumulation of phenolics, flavonoids, condensed tannins and volatiles metabolites in the *in vitro* regenerated microshoots. The effect of these two elicitors on the antioxidant potentials of tree wormwood extracts was also evaluated.

2. MATERIALS AND METHODS

2.1. Plant material and *in vitro* regeneration conditions

The regeneration of tree wormwood microshoots was achieved following the direct *in vitro* micropropagation protocol previously developed for this species (Riahi et al., 2022). Fresh nodal segments (2 cm) originated from a healthy mother plant growing under field conditions were used to initiate axenic *in vitro* cultures. The explants were surface sterilised with 70% ethanol (1 min), then treated during 20 min in a solution of sodium hypochlorite (10%) and Tween 20 (0.01%). Afterword, the nodal segments were washed five times with sterile distilled water then dried on sterile filter paper.

The nodal segments were inoculated in test tubes containing 15 mL of free-phytohormone Murashige and Skoog culture medium (Murashige & Skoog, 1962), 3% sucrose, 0.8% agar and adjusted pH to 5.8. After the development of axenic microshoots, the subcultures were achieved in glass vials containing 50 mL of MS culture medium supplemented with the two phytohormones 6-benzylaminopurine (0.5 mg/L) and indole 3-acid acetic (0.1 mg/L). To allow the development of *A. arborescens* L. microshoots, the glass vials were maintained in a growth room under controlled conditions (cool-white fluorescent lamps, photoperiod: 16/8, temperature: 25±1°C). One-month old developed microshoots were used for *in vitro* elicitation experiments.

2.2. Treatments application

One-month old microshoots of the studied species were transferred to MS culture medium supplemented with 100 mM NaCl or KCl. A group of micropropagated shoots was transferred to basic MS culture medium and was used as a control. Each *in vitro* culture condition was performed with 12 replicates. The plant material was harvested after three days (72 hours) of the application of the elicitors.

2.3. Phenolic compounds contents

The *in vitro* regenerated microshoots of *A. arborescens* L. (Figure 1) were uniformly ground to serve for phytochemical analyses. The ethanolic extract was prepared by adding one gram of each sample to 10 mL of 80% ethanol. A dynamic maceration in a rotary shaker (120 rpm) during two hours was employed to extract the phenolic compounds. The obtained ethanolic extracts were filtered through Whatman paper No. 1 then stored at 4°C in the dark until further assays.

The contents of total polyphenols in the ethanolic extracts of tree wormwood microshoots were quantified following the Folin-Ciocalteu method (Singleton & Rosi, 1965). In brief, 125 μL of diluted extract and 625 μL of 10% Folin-Ciocalteu reagent were mixed. After standing for 5 minutes in the dark, 500 μL of 7.5% sodium carbonate solution (Na_2CO_3) were added. The absorbance of each reaction was read at 760 nm, after incubation (30 minutes) in the dark. The total polyphenols amounts were estimated using a calibration curve of gallic acid (0-200 μg/mL). The contents of phenolic compounds were expressed as mg gallic acid equivalent per gram of fresh weight (mg GAE/g FW).

The contents of flavonoid compounds in the ethanolic extracts of *A. arborescens* L. microshoots were determined using the aluminum chloride colorimetric method (Djeridane et al., 2006). For each sample, one mL of adequately diluted extract was added to one mL of $AlCl_3.6H_2O$ solution (2%). The mixture was incubated 15 minutes in the dark then the absorbance was measured at 430 nm. A calibration curve using rutin (0-200 μg/mL) as standard was used to deduce the concentrations of flavonoids. The flavonoid contents in the investigated extracts were expressed as mg rutin equivalent per gram of fresh weight (mg RE/g FW).

The vanillin method was used to quantify the condensed tannin contents in the studied extracts (Sun et al., 1998). After the preparation of the adequate dilution for each ethanolic extract, 50 μL were mixed with 750 μL of freshly prepared vanillin solution (4%) and 375 μL of concentrated H_2SO_4. The absorbance of the reaction mixture was measured at 500 nm after 15 min of incubation in the dark. The amounts of condensed tannins were calculated using a calibration curve of catechin (0-200 μg/mL) and given as milligrams of catechin equivalent per gram of fresh weight (mg CE/g FW).

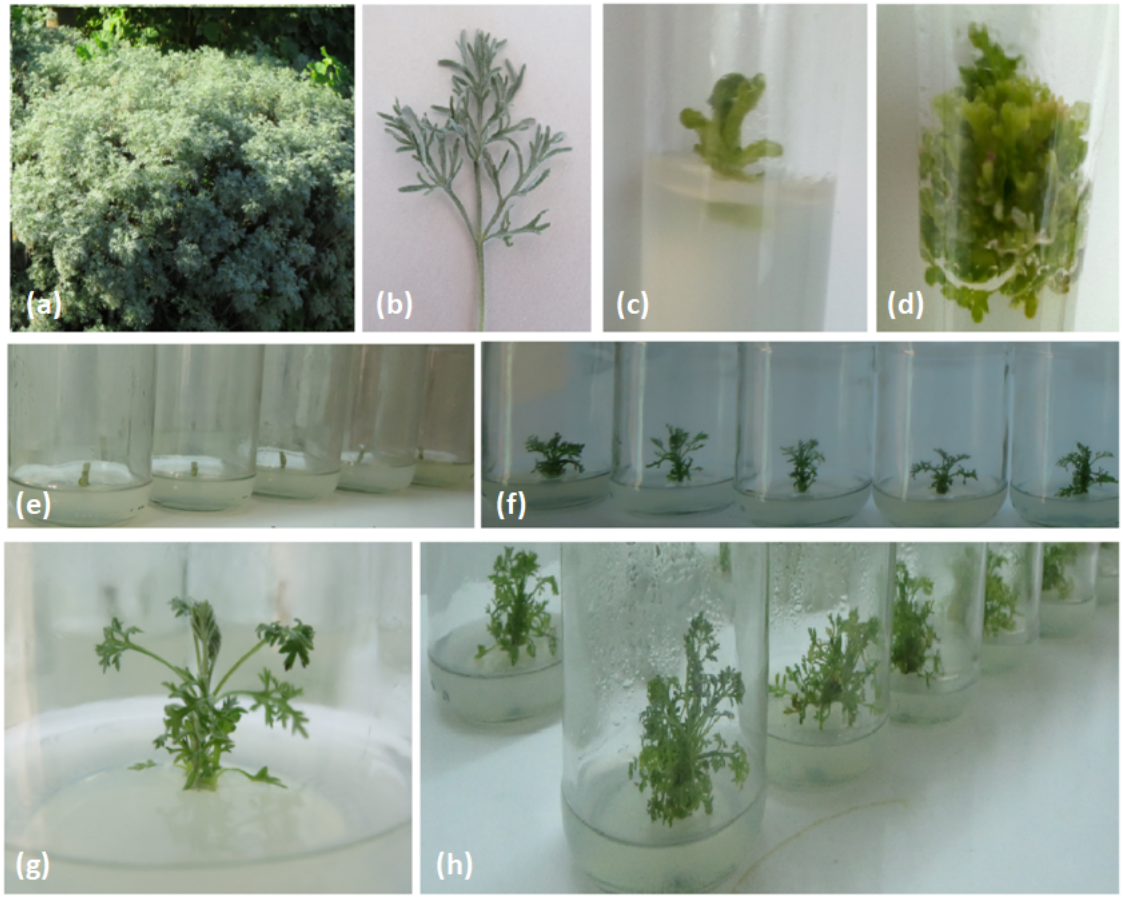

Figure 1. Micropropagation of tree wormwood using nodal segments as explants. a–d: initiation of axenic culture, e–h: *in vitro* regeneration of microshoots in MS medium supplemented with 0.5 mg/L BAP and 0.1 mg/L IAA.

2.4. Volatile metabolites contents

The volatile compounds were extracted using hexane solvent following the method described by Chograni et al. (2010) with some modifications. The plant material is frozen (24 hours) then ground in a mortar with a volume of hexane (hexane volume (mL) = plant material mass (g) x 10) and stirred 12 hours in a rotary shaker (120 rpm) in the dark. Afterward, the extracts were filtered using Whatman paper No. 1 then through a 0.45 μm syringe filter. The extraction yields (%) were estimated based on the fresh weight of plant material.

2.5. Antioxidant activity

The radical scavenging potential of tree wormwood extracts was conducted based on the DPPH test (Hanato et al., 1988). For each sample, one mL of tree wormwood extract, at different concentrations, was mixed with 0.5 mL of 0.2 mM DPPH solution freshly prepared. The mixture was allowed to stand in the dark for 30 min at room temperature then the absorbance was recorded at 517 nm. The absorbance of a reaction without extract considered as a control was measured. The percentage of inhibition of each sample was determined as PI = [(Absorbance control - Absorbance extract)/ Absorbance control] * 100. The IC_{50} value (μg/mL) defined as the required concentration of each extract able to inhibit 50% of DPPH radicals was calculated for each sample. The radical scavenging potential of butylated hydroxytoluene (BHT), used as a standard antioxidant, was estimated in the same conditions as for the extracts.

3. DATA ANALYSES

All the achieved analyzes were assessed in three replicates. The results are presented as means values ± standard variations. The quantitative variation of the studied parameters was assessed applying the variance analysis. The Duncan's test was used to classify the means values at a significance level of 0.05. These data analyses were achieved using the software IBM SPSS version 28.0.

4. RESULTS

4.1. Variation of phenolic contents

The amounts of total polyphenols, flavonoids and condensed tannins showed significant variations between the treated microshoots and the control condition (Figure 2). The application of 100 mM NaCl or KCl to tree wormwood *in vitro* cultures exhibited positive effect on the accumulation of total

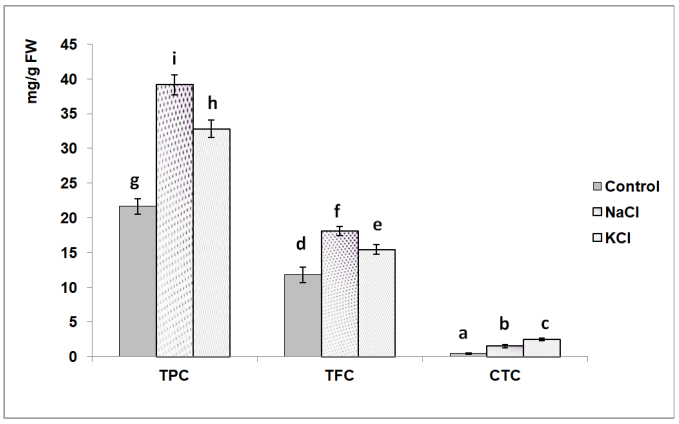

Figure 2. Variation of the total polyphenols (mg GAE/g FW), flavonoids (mg RE/g FW) and condensed tannins (mg CE/g FW) contents among the treated and untreated microshoots.

polyphenols in the treated microshoots. A significant increase in the total polyphenols concentrations over the untreated vitroplants (21.64 ± 1.09 mg GAE/g FW) was revealed with both elicitors NaCl (39.16 ± 1.45 mg GAE/g FW) and KCl (32.78 ± 1.26 mg GAE/g FW). The application of these two abiotic elicitors resulted in the enhancement of the accumulation of phenolic compounds with improvement rates over the control around 80% and 51 % for NaCl and KCl, respectively.

The total flavonoid contents vary as the same way as for the total polyphenols and showed significant increases for the treated vitroplants compared to the control. The application of NaCl resulted in the highest levels of flavonoids (18.09 ± 0.63 mg RE/g FW), followed by KCl treatment (15.44 ± 0.65 mg RE/g FW). The lowest levels were recorded for the untreated microshoots (11.81 ± 1.12 mg RE/g FW). The improvement of the concentration of flavonoids in the aerial parts of *A. arborescens* L. following the application of the two elicitors for 72 hours is about 53% with NaCl and 30% with KCl treatments.

The effect of abiotic elicitation on the biosynthesis of condensed tannins is more important. The untreated microshoots presented lower contents of condensed tannins (0.46 ± 0.10 mg CE/g FW). The amounts of condensed tannins increased significantly with the application of the two elicitors and reached 2.46 ± 0.18 (mg CE/g FW) with the application of KCl and 1.51 ± 0.21 (mg CE/g FW) with NaCl treatment. The improvement rates of the contents of condensed tannins with KCl and NaCl are about 430% and 226%, respectively.

4.2. Volatile metabolites contents

The variation of the volatile metabolites contents among the treated and untreated vitroplants was analyzed. The obtained results showed a significant increase in the amounts of volatile metabolites in the elicited microshoots compared to the control for the two elicitors (Figure 3). The obtained findings showed that the highest volatile metabolites content was recorded upon KCl treatment (0.95 ± 0.04%) with an improvement rate over the control (0.68 ± 0.03%) of about 39%. A volatile metabolites content of 0.84 ± 0.03 % was observed following the application of NaCl giving an improvement rate of about 23% compared to the untreated vitroplants.

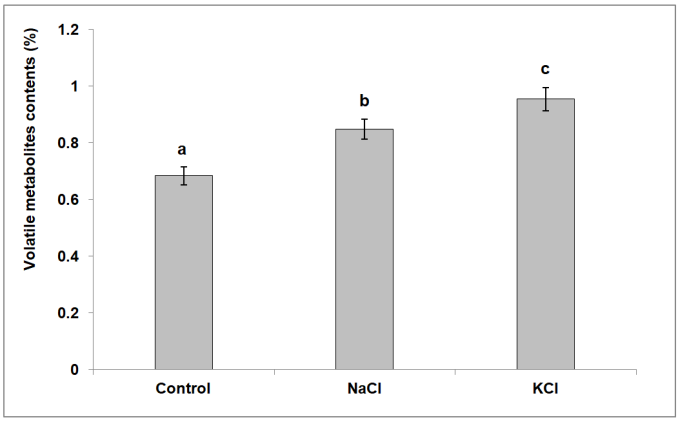

Figure 3. Variation of volatile metabolites contents (%) among the three studied experimental conditions.

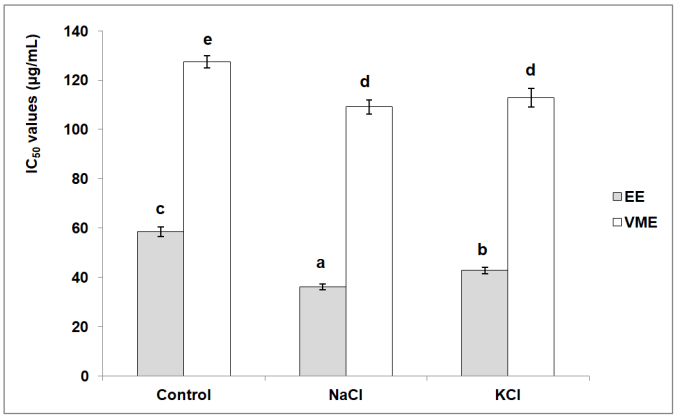

Figure 4. Variation of IC_{50} values (μg/mL) based on DPPH test of the studied extracts of tree wormwood microshoots.

4.3. Antioxidant activities

The antioxidant activities of the ethanolic and volatile metabolites extracts of *Artemisia arborescens* L. microshoots without and after elicitation with NaCl or KCl were estimated applying the DPPH test. A significant decrease of the IC_{50} values of the extracts of treated microshoots with NaCl or KCl compared to the control was observed (Figure 4). The application of NaCl gave the highest antioxidant activity (IC_{50} = 36.24 ± 1.15 μg/mL) followed by KCl treatment (IC_{50} = 42.87 ± 1.24 μg/mL) while the lowest antioxidant activity was recorded for the control (IC_{50} = 58.54 ± 1.99 μg/mL). Thus, the improvement of the antioxidant power of ethanolic extracts of tree wormwood microshoots treated for three days with 100 mM NaCl or KCl is about 38% and 26%, respectively.

Compared to the antioxidant standard BHT (IC_{50} = 22.67 ± 0.93 μg/mL), the antiradical capacities of the ethanolic extracts of treated vitroplants are important. Lower antioxidant

potential was recorded for the volatile metabolites extracts of *A. arborescens* L. compared to its ethanolic extracts. The volatile metabolites extract of untreated microshoots showed higher IC_{50} value (127.39±2.55 μg/mL) than those treated with NaCl (109.10 ± 2.92 μg/mL) or KCl (112.83 ± 3.80 μg/mL) elicitors which exhibited higher antioxidant potential. It is noted that the treatment with NaCl or KCl led to the enhancement of the scavenging activity of about 14% and 11%, respectively. No significant variation for IC_{50} values was observed between the volatile metabolites extracts obtained with NaCl or KCl elicitors.

5. DISCUSSION

The effects of NaCl and KCl (100 mM), applied as two abiotic elicitors, on the production and bioactivity of secondary metabolites in *A. arborescens in vitro* regenerated microshoots was carried out. The obtained findings highlighted the effectiveness of the exogenous application of these two abiotic elicitors on the enhancement of polyphenolic, flavonoids, condensed tannins and volatile metabolites contents in the treated vitroplants. NaCl elicitor showed higher improvement rates on the *in vitro* accumulation of total polyphenols (80%) and flavonoids (53%) while KCl elicitor exhibited higher positive effect on condensed tannins (430%) and volatile metabolites (39%) contents.

NaCl and KCl salt elicitors were used for the induction and improvement of the accumulation of secondary metabolites in various plant species under *in vivo* production systems. The recorded responses of plant cultures to these two abiotic elicitors showed significant variation depending on genetic factor, type, exposure time and concentration of elicitors. Higher total phenolics, total flavonoids and DPPH radical scavenging activity over the control were reported for *Stevia rebaudiana in vitro* shoot and call cultures upon application of 100 mM NaCl (Javed & Gürel, 2019). The application of 100 mM NaCl for six days enhanced significantly the alkaloid solasodine concentration in the hairy roots of *Solanum khasianum* by fourfold (Srivastava et al., 2016). The last authors also reported that one week application of 100 mM NaCl increased the accumulation of the alkaloid ajmalicine up to 14.8-fold in hairy root cultures of the medicinal species *Rauwolfia serpentina*.

In *Digitalis purpurea* L. *in vitro* shoot cultures, while NaCl didn't show significant effect, KCl enhanced the accumulation of the two glycosides digitoxin (at 80 mM) by 7.75-folds and digoxin (at 200 mM) by 8.7-folds (Patil et al., 2013). Furthermore, the concentration of the bioactive terpenoid bacoside A in the *in vitro* shoot cultures of *Bacopa monnieri* was enhanced significantly over the control upon treatment with 100 mM KCl (Ahire et al., 2014). Potassium chloride application at high level (800 mM) inhibited the growth of cell suspension cultures of *Ginkgo biloba*, however a significant increase of the terpenoids bilobalide and ginkgolides concentrations was reported at this concentration (Kang et al., 2010). Other report interested in the medicinal species *Salvia mirzayanii* showed that moderate salinity increase the synthesis of total phenolics, volatile components and DPPH scavenging activity (Valifard et al., 2014).

High salt concentrations have generally negative effects on the biomass and growth parameters as they induce both ionic and osmotic stress in plants (Ahire et al., 2014; Lamaoui et al., 2019). Thus, the optimal concentration of salt elicitor that may induce or increase the production of specific secondary metabolites without exhibiting negative growth effects should be determined for each applied elicitor and treated plant species. The qualitative and quantitative enhancement of secondary metabolites production under induced stress by abiotic elicitors is the result of various genomic and biochemical complex mechanisms including the stimulation or the inhibition of tolerance related genes, proteins phosphorylation/dephosphorylation, enzymes expression which resulted in the activation of the biosynthetic pathways of specific secondary metabolites included in the tolerance response (Golkar et al., 2020; Narayani & Srivastava, 2017).

The obtained results in this study confirm previous investigations which reported the enhancement of the antioxidant activities of *in vitro* cultures upon the application of various types of abiotic stress (Lamaoui et al., 2019).The observed increase in the antioxidant potentials of the elicited microshoots of *A. arborescens* L. could be explained by their higher contents in secondary metabolites compared to the untreated microshoots. Indeed, faced to the induced abiotic stress the plant cells acted through the accumulation of specific secondary metabolites such as phenolic acids and favonoids to reduce damage caused by oxidative stress. The increase in polyphenol and total flavonoid contents resulted in the enhancement of the antioxidant capacities of the extracts (Riahi et al., 2020). Higher plants have the ability to modify their secondary metabolism in many ways in response to different types of arising abiotic stress (Isah, 2019).

The use of elicitation as an improvement tool for the biotechnological production of plant secondary metabolites presented wide applications in the last decade and seems as a promising approach especially for medicinal plant species showing limits in conventional propagation or threatened by overexploitation. *In vitro* production of secondary metabolites by *in vitro* cell, tissue and organ cultures stimulated by abiotic and biotic elicitors presents many advantages compared to conventional field growing system namely the absence of fluctuations of environmental factors, the continuous supply of high quality bioactive molecules with considerable amounts and in some cases the accumulation of new compounds (Jirakiattikul et al., 2020). While the efficacy of elicitation was confirmed by several studies under *in vitro* and *in vivo* production system, the detected alteration in secondary metabolism of aromatic and medicinal species was reported to be a very complex process and needed to be clarified.

6. CONCLUSION

The application of 100 mM NaCl or KCl used as abiotic elicitors, to one-month old microshoots of *Artemisia arborescens* L. during 72 hours resulted in the enhancement of the accumulation of total polyphenols, flavonoids, condensed tannins and volatile metabolites. Furthermore, the application of these two abiotic elicitors enhanced the antioxidant potential of the ethanolic and volatile metabolites extracts of the treated *in vitro* regenerated microshoots. The obtained findings confirmed the efficiency of abiotic elicitation as an alternative strategy to increase the concentrations of bioactive secondary metabolites under *in vitro* production system. Further investigations are required to optimize the experimental protocols and to elucidate the molecular mechanisms implicated in the response of vitroplants to the applied elicitors. These investigations will allow better management of the production of these compounds with increasing demand in agrochemical, pharmaceutical, cosmetic and food industries.

CONFLICTS OF INTEREST

All authors declare that there is no conflict of interest.

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AUTHOR CONTRIBUTIONS

LR - Research concept and design, FBR, HC, LR - Collection and/or assembly of data, FBR, HC, MBR, LR - Data analysis and interpretation, FBR, MBR, LR - Writing the article, LR - Critical revision of the article, FBR, HC, MBR, LR - Final approval of the article.

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