# ANTIOXIDANT AND ANTIMICROBIAL POTENTIAL OF *ACHILLEA OXYLOBA* SSP. *SCHURII* (SCH. BIP.) HEIMERL (*ASTERACEAE*) CALLUS *IN VITRO*

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*Achillea oxyloba* ssp. *schurii* (Sch.Bip.) Heimerl (*Asteraceae* family) is an endemic species of the Eastern and Southern Carpathians, being restricted to the alpine and subalpine belt, in rocky places or the vicinity of the streams. No reports are available regarding the induction of callus to *Achillea oxyloba* ssp. *schurii* (Sch.Bip.) Heimerl (*Asteraceae* family) species and determination of antioxidant and antimicrobial activity to these *in vitro* obtained formations and also *in vitro* multiplication. Callus cultures were initiated from nodal segments of the potted plant cultured on Murashige and Skoog (MS) medium supplemented with plant growth factors in five different variant concentrations. Optimal composition of *in vitro* culture media for multiplication and improving biological activities of the callus clumps was determined. *Achillea oxyloba* ssp. *schurii* (Sch.Bip.) Heimerl *in vitro* induced calli can synthesize important secondary metabolites like phenolics. We determined antioxidant activity and total phenolic content of potted *Achillea schurii* shoots and callus obtained on different culture media composition. The polyphenolic content in callus samples significantly exceeded the polyphenolic content of the potted plant. Two samples of the callus formations were subjected to antimicrobial tests at different growth stages, first developed on induction callus medium, at 4 weeks, and the second, developed on the callus propagation medium, at 5 weeks.

The percentage of bacterial reduction compared to the positive control was investigated for both samples, against *Staphylococcus aureus* ATCC 25923, after 4 and 24 hours of incubation. The results of the experiment showed that the bacterial reduction was more evident in sample 1 after 4 hours of contact of the callus with the bacterial strain. Assessing bacterial reduction capacity of the callus formations revealed employed method efficiency. Further studies intend to level up the polyphenol content of these structures, to improve their bioactive properties by modulating *in vitro* culture medium composition and *in vitro* conditions.

**Keywords:** *Achillea schurii* bioactive properties, *in vitro* callus culture, polyphenols.

## INTRODUCTION

*Achillea oxyloba* ssp. *schurii* (Sch.Bip.) Heimerl (*Asteraceae* family) is an endemic species of the Eastern and Southern Carpathians, being restricted to the

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alpine and subalpine belt, in rocky places or the vicinity of the streams. First mentioned in 1856 as *Achillea schurii* (Schultz 1856), the species was considered at times to be an independent species, but the analysis of morphological characters placed it as subspecies of *A. oxyloba*. The cariological studies (Tăcina 1979) confirmed diploidy and the age of the taxon (paleoendemic). The species (Fig. 1) belongs to the Ptarmica Section (Neck.) Koch and it can be distinguished by solitary stems, 10–20 cm high, foliated, sparsely pubescent, usually unbranched, and with only one (2–3) capitula; the leaves are 3–4.5 cm long, 2–3 pinnatisect, elongated, with the uppermost lobes 0.5–1 mm wide, cartilaginous mucronate apex. Capitula 10–15 mm wide; phyllaries ovate elongated, sparsely pubescent, obtuse, with denticulate apex and dark brown border along the margins. Flowers 10–15, ligules white, elliptical, 6–9 mm long, slightly crenated. Achenes are convex 2–2.5 cm long. Bloom July to August (Prodan and Nyarady 1964; Ciocarlan 2009, Sârbu *et al.* 2013). It is included in the national red list of vascular plants (Oltean *et al.* 1994).

Fig. 1. *Achillea oxyloba* ssp. *schurii* (Sch.Bip.) Heimerl in Piatra Craiului, 1,740 m altitude (IV).

So far no studies have been conducted regarding *in vitro* morphogenetic potential of this species. Improving existing, and developing new effective conservation strategies are necessary. Advances in biotechnology provide new methods for plant diversity conservation and evaluation (Manole–Păunescu 2014). Also, plant biotechnologies can improve *ex situ* classical strategies providing new options for collection, multiplication, and plant biodiversity conservation. Biotechnological methods can help *in situ* measures providing an alternative for *ex-situ* plant conservation (Cruz–Cruz 2013, Holobiuc *et al.* 2018).

No reports are available regarding the induction of callus to *Achillea oxyloba* ssp. *schurii* (Sch.Bip.) Heimerl (*Asteraceae* family) species and determination of

antioxidant and antimicrobial activity to these *in vitro* obtained formations and also *in vitro* multiplication. According to Effert (2019), the totipotency of the callus cultures triggers the biosynthesis of secondary metabolites, by the full genetic information from the origin plant. The related species have been extensively studied for *in vitro* multiplication (Turker *et al.* 2009, Shatnawi 2013, Marcu *et al.* 2014, Cosac 2016). Biochemical and antimicrobial potential of *Achillea oxyloba* ssp. *schurii* (Sch.Bip.) Heimerl species have been studied on flowers extract (Benedec 2016). These studies highlight that *Achillea oxyloba* ssp. *schurii* (Sch.Bip.) Heimerl is an important source of bioflavonoids, with significant therapeutic potential.

The aim of our study was to investigate the antimicrobial and antioxidant activities of *Achillea oxyloba* ssp. *schurii* (Sch.Bip.) Heimerl callus formations *in vitro* obtained and to determine quantitatively the phenolic content.

## MATERIALS AND METHODS

### PLANT MATERIAL, INDUCTION, AND MULTIPLICATION OF CALLUS CULTURES

The sampling area was represented by Piatra Craiului, Padinile Frumoase 1,740 m, altitude. The plant material was collected in July 2018 in the blossom period. Nodal segments were used to initiate *in vitro* callus culture. These explants were surface – disinfected in 70% (v/v) ethanol for 30s, then with 0.5% dichloroisocyanuric acid sodium salt for 3 min, followed by three rinses in sterile distilled water. The pH of the medium was adjusted to 5.8 before autoclaving.

The basal culture medium used was Murashige and Skoog (MS) (Murashige & Skoog 1962) with 8% (w/v) agar as a gelling agent and 3% (w/v) sucrose. To the basal culture medium, we added 0.5 mg $L^{-1}$ 6-benzylaminopurine (BAP) in combination with 0.1 mg $L^{-1}$ indole-3-acetic acid (IAA) – V1 variant to promote callus induction from internodal cuttings.

To determine the optimal composition of *in vitro* culture media for multiplication and improving their biological activities, the callus clumps were transferred to a medium containing the following variants: V2 5 mg $L^{-1}$ kinetin and 1 mg $L^{-1}$ indole-3-butyric acid (IBA) MS medium, V3 3 mg $L^{-1}$ 6-benzylaminopurine (BAP), 0.5 mg $L^{-1}$ IAA and 20 g $L^{-1}$ sucrose, V4 2.25 mg $L^{-1}$BAP, 0.18 mg $L^{-1}$ IAA and 20 g $L^{-1}$ sucrose.

In the first stage of the experiment, the cultures were incubated at 21°C in the dark (the first three days), then with a 16h daylight of 30 µmol $m^{-2}$ $s^{-1}$. Callus explants were cultured to a fresh medium every 2 weeks. The photoperiod of maintaining cultures was changed subsequently to 18 h with an illumination of 45 µmol $m^{-2}$ $s^{-1}$. Callus formations at two different growth stages were subjected to antimicrobial tests (Fig. 3). Further investigations consisted of biochemical analyses of 5 variants (V1–V4, IV) of calluses and original plant. Samples were collected for analysis periodically.

## EVALUATION OF THE ANTIBACTERIAL ACTIVITY OF *ACHILLEA SCHURII* CALLUS

The Gram-positive bacterium *Staphylococcus aureus* ATCC 25923 was selected to test the antibacterial activity of *Achillea schurii* callus (Fig. 3, Fig. 7). The bacterial strain was grown aerobically at 37°C for 18h in Luria-Bertani (LB) broth. The growth medium had the following composition (g/L): tryptone 10, yeast extract 5, sodium chloride 10, and agar 15. The pH of the growth media was adjusted to 7.2 before sterilization (121°C for 20 minutes). 1 g of vegetable material was weighed and cut into small pieces and distributed into a sterile Erlenmeyer flask. To conduct the test, 25 mL of LB liquid growth medium were added and inoculated with 0.5 mL of a standardized microbial suspension obtained by adjusting the turbidity of bacterial culture to 0.5 McFarland. A control sample, represented by the bacterial culture, was also prepared. After 4h and 24h from the contact of the bacterial cells with the vegetable material, the samples were tested for antibacterial activity by determining the number of colony-forming units per milliliter (CFU/mL) in relation to the positive control. For all samples, serial decimal dilutions were performed and 100 µL of them were inoculated by pour plate method. The plates were incubated at 37°C for 24h and the colonies were observed. The experiment was made in duplicate. The percentage of bacterial reduction was calculated according to the equation: (B-A)/B x 100, where: B = CFU/mL of the positive control; A = CFU/mL of the samples.

## BIOCHEMICAL ANALYSES

The material used for biochemical analyses was represented by *in vitro* callus of *Achillea schurii* obtained on different culture media (Fig. 2–5) and whole aerial parts (excluding flowers) of potted plants. The plant material was ground with quartz sand, 100% *methanol* was added and the mixture was let for extraction at room temperature for *24 hours*. The supernatant obtained by centrifugation at 10,000 G for 20 minutes was used for subsequent analysis.

## ANTIOXIDANT CAPACITY DETERMINATION (DPPH METHOD)

The antioxidant activity was determined using the method described by Marxen *et al.* (2007). The reaction mixture consisted of 100 µl extract, 2.25 ml methanol, and 150 µl 1,27mM DPPH in experimental variants, while in control the extract was replaced with methanol. The antioxidant capacity has been reading at 515 nm, 30 minutes after incubation at room temperature, representing differences between standard control Trolox (synthetic antioxidant α-tocopherol analog) and samples values.

TOTAL PHENOLIC CONTENT (TPC) DETERMINATION

For TPC determination, except for minor changes, the method proposed by Mihailovic *et al.* (2013) was employed. The reaction mixture (0.5 ml of extract, 2.5 ml of Folin-Ciocalteu reagent, and 2 ml of 7.5% $Na_2CO_3$) was let for 30 minutes at room temperature, and absorbance was read at 765 nm. The total phenolic content, expressed as gallic acid equivalents / fresh weight (EAG µg/g sample) represented the average of three repetitions.

## RESULTS AND DISCUSSIONS

INDUCTION AND PROPAGATION OF CALLUS CULTURE

A procedure of callus induction from nodal segments was developed for *Achillea oxyloba* ssp. *schurii* (Sch.Bip.) Heimerl. No investigations have been carried out on *in vitro* regeneration to *Achillea oxyloba ssp. schurii* (Sch.Bip.) Heimerl species, nor on callus or via callus. 15–17 d after inoculation, callus formations were developed at the explant edges (Fig. 2) and gradually covered an increasing surface on medium containing 0.1 mg $L^{-1}$ IAA and 0.5 mg $L^{-1}$ BAP.

Initially, callus obtained by us was white in color with a compact structure (Fig. 2). Promoting cell expansion in plant tissue culture, the type and concentration of cytokinin significantly determined the callusogenic potential of nodal explants.

The complete changes in the proportion and type of auxin and cytokinin in the variant 2 were conducted to an intense dark green color of the callus (Fig 4). In the subsequent days of culture primary calluses gradually converted into homogenous masses, with a compact texture, of different colors and shades, namely dark green (Fig. 4), white and green (Fig. 5), yellowish-green, and morphogenetic (Fig. 6). The V3 and V4 media, which were supplemented with the same type of hormones, but with concentrations finely modulated, were effective for the induction of nodular (Fig. 5) to organogenic callus (Fig. 6), characterized by organogenic structures, namely, shoot and foliar primordia formations. Combinations of BAP and IAA were found most effective for callus induction and multiplication. Turker *et al.* (2009) mention the first report on plant regeneration system for shoot multiplication to *Achillea millefolium* L. with an effective protocol based on concentrations of 3.0 mg/l $^{-1}$ BAP and 0.5 mg//l $^{-1}$ IAA.

Supplementing within 20 g $L^{-1}$ sucrose this combination, we obtained the best results in callogenesis production to our species of interest. Surprisingly, no hyperhydricity appeared in the *Achillea oxyloba ssp. schurii* (Sch.Bip.) Heimerl callus culture, in contrast to the other species studied by us, like *Ocimum basilicum* L., in the early stages.

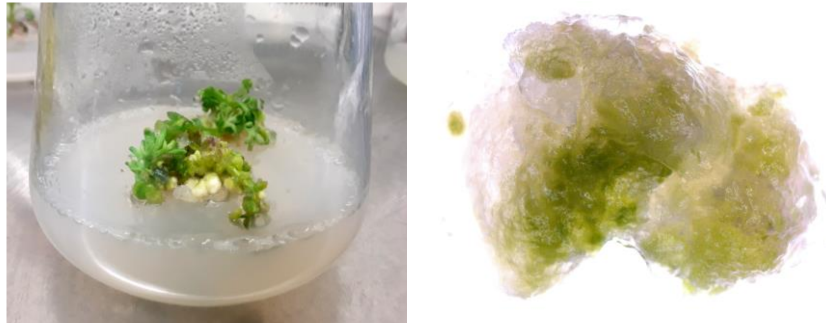

Fig. 2. a. White–green callus formations at the shoots base. MS medium with 0.1 mg $L^{-1}$ 3-indole acetic acid (IAA) and 0.5 mg $L^{-1}$ benzyl adenine (BAP); b. image of the callus (0.63x) at stereo microscope Stemi 2000 - C.

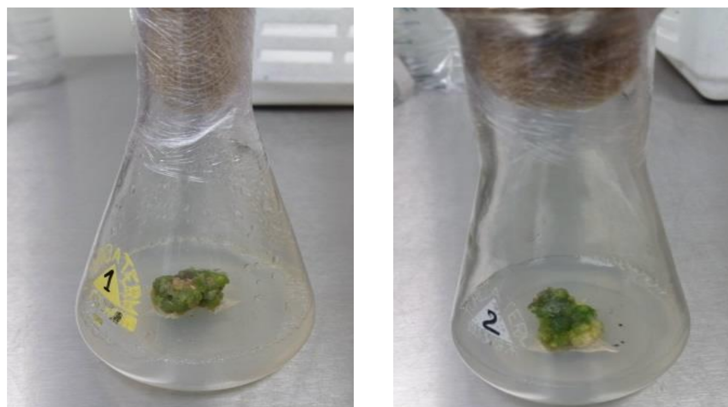


Fig. 3. (V1) *Achillea schurii* callus:
a. Sample 1 (left) (4 weeks) – callus developed on 0.1 mg $L^{-1}$ indole acetic acid (IAA) and 0.5 mg $L^{-1}$ benzyl adenine (BAP) supplemented MS medium.
b. Sample 2 (right) (5 weeks) – morphogen callus developed on 0.5 mg $L^{-1}$ indole acetic acid (IAA) and 3 mg $L^{-1}$ benzyl adenine (BAP) supplemented MS medium.

Fig. 4. (V2) Callus obtained on 5 mg $L^{-1}$ k and 1 mg $L^{-1}$ indole-3-butyric acid (IBA).

Fig. 5. (V3) Developed callus in MS medium containing 0.5 mg $L^{-1}$ 3-indole acetic acid (IAA), 3 mg $L^{-1}$ benzyl adenine (BAP), and 20 g $L^{-1}$ sucrose.

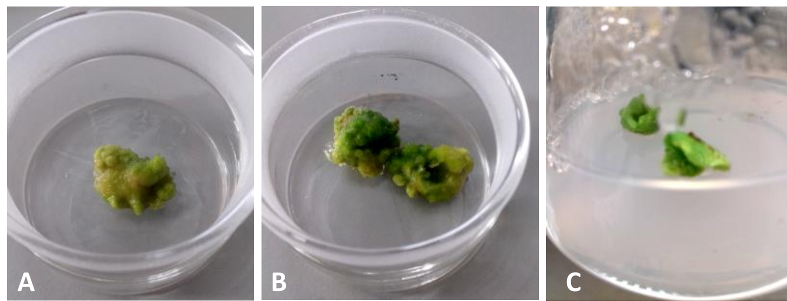


Fig. 6. (V4) Green yellow callus with shooting buds (A) and foliar primordia (C) on 0.18 mg $L^{-1}$ 3-indole acetic acid (IAA) and 2.25 mg $L^{-1}$ benzyl adenine (BAP) with 20 g $L^{-1}$ sucrose.

ANTIBACTERIAL ACTIVITY OF *ACHILLEA SCHURII* CALLUS

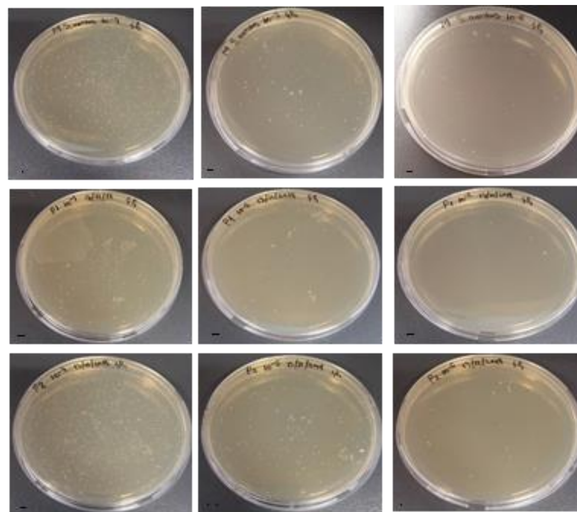

Fig. 7. *S. aureus* ATCC 25923 colonies after 4 hours of contact of the callus with the bacterial strain. Positive control (A: $10^{-4}$ dilution, B: $10^{-5}$ dilution, C: $10^{-6}$ dilution); Sample 1 (D: $10^{-4}$ dilution, E: $10^{-5}$ dilution, F: $10^{-6}$ dilution); Sample 2 (G: $10^{-4}$ dilution, H: $10^{-5}$ dilution, I: $10^{-6}$ dilution).

The obtained results of antibacterial activity of *Achillea schurii* callus against *Staphylococcus aureus* ATCC 25923 are shown in Fig. 7. The percentage of bacterial reduction for sample 1 was 70.6% after 4 hours of contact of the callus with the bacterial strain (Table 1), but no prolonged inhibitory activity was observed. The decrease in antimicrobial activity of sample 1 after 24 hours of incubation compared to that after 4 hours of incubation can be explained as a consequence of the limited antibacterial activity of the callus, affecting only in the early hours of microbial growth.

In the case of sample 2, no inhibition growth was observed after 4 hours of exposure to callus. When a longer incubation was applied, the bacterial reduction increased slightly (22.2%), compared with the control sample.

*Table 1*

Antibacterial activity of *Achillea schurii* callus

| | CFU/mL | 4h<br>Percentage of bacterial reduction compared to the positive control | CFU/mL | 24h<br>Percentage of bacterial reduction compared to the positive control |
|---|---|---|---|---|
| Positive control | $1.38 \times 10^8$ | | $4.5 \times 10^9$ | |
| Sample 1 | $4.05 \times 10^7$ | 70.6% | $4.5 \times 10^9$ | 0% |
| Sample 2 | $1.39 \times 10^8$ | 0% | $3.5 \times 10^9$ | 22.2% |

ANTIOXIDANT ACTIVITY

The highest antioxidant activity was determined in callus grown on the V3 medium variant, closely followed by extracts obtained from potted plants, but the difference between the two variants is not statistically significant. Callus grew on V1, V2, and V4 media variants showed significantly lower values of antioxidant activity (51–89 mM Trolox/g fresh weight) than V3 variant and donor plant (IV variant).

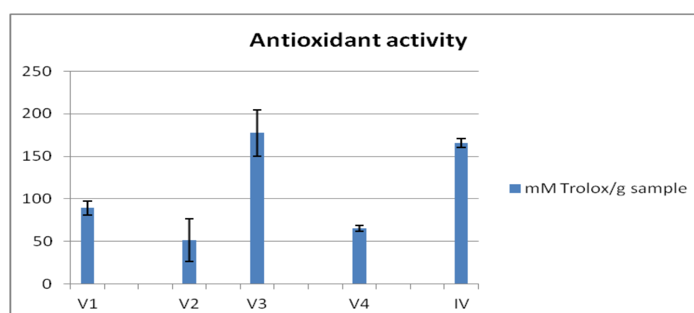


Fig. 8. Antioxidant activity of potted *Achillea schurii* shoots (IV) and callus obtained on different culture media (V1: MS medium supplemented with 0.1 mg $L^{-1}$ IAA and 0.5 mg $L^{-1}$ BAP; V2: MS medium supplemented with 1 mg $L^{-1}$ IBA and 5 mg $L^{-1}$ k; V3: MS medium supplemented with 0.5 mg $L^{-1}$ IAA, 3 mg $L^{-1}$ BAP and 20 g $L^{-1}$ sucrose V4: MS medium supplemented with 0.18 mg $L^{-1}$ IAA and 2.25 mg $L^{-1}$ BAP with 20 g $L^{-1}$ sucrose).

Total phenolic content determinations revealed that the V3 medium variant induced the highest polyphenols synthesis. However, an insignificant difference is noted between callus grown on V3 and V4 media, but the polyphenolic content in both samples significantly exceded the polyphenolic content of potted plants. Callus grown on V1 and V2 media had the lowest content in polyphenols.

The results showed that *Achillea oxyloba* ssp. *schurii* (Sch.Bip.) Heimerl *in vitro* induced calli can synthesize important secondary metabolites like phenolics. Growth media composition influenced the polyphenols content, sucrose concentration of 20 g $L^{-1}$, and phytohormones concentrations of 0.5 mg $L^{-1}$ IAA, 3 mg $L^{-1}$ 6-benzylaminopurine BAP, from V3 and V4 stimulating the polyphenols synthesis in *Achillea oxyloba* ssp. *schurii* (Sch.Bip.) Heimerl *Achillea schurii* callus. Our results are in agreement with other studies that approached secondary metabolites content *in vitro* cultures with a maximum of flavonoids and phenolics concentration and antioxidant activities registered in cultures grown on media supplemented with sucrose (Fazal *et al.* 2016) in shake-flask suspension cultures of *Prunella vulgaris* or with sucrose and maltose (Ali *et al.* 2016) in cell suspension cultures of *Artemisia absinthium* L. Overall, although it is not completely correlated with antioxidant activity, the polyphenolic content in *A. schurii* callus was higher comparing with a potted plant. Our media variants for biochemical investigations were established and conducted according to the results obtained in microbiological investigations.

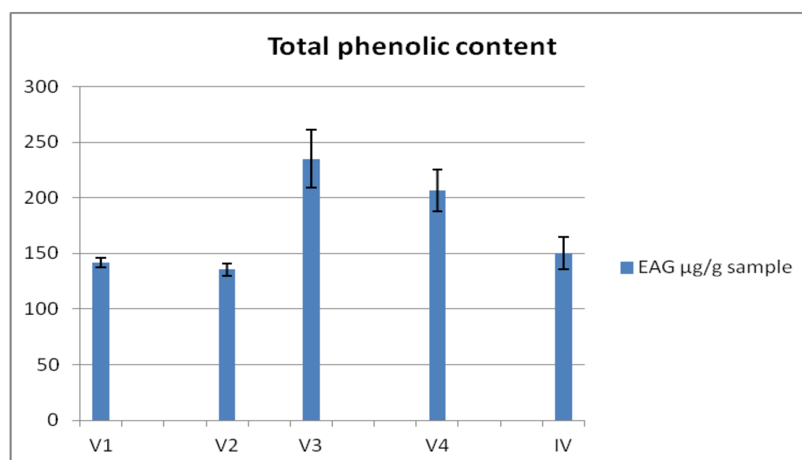


Fig. 9. Total phenolic content of potted *Achillea schurii* shoots (IV) and callus obtained on different culture media (V1: MS medium supplemented with 0.1 mg $L^{-1}$ AIA and 0.5 mg $L^{-1}$ BAP; V2: MS medium supplemented with 5 mg $L^{-1}$ k and 1 mg $L^{-1}$ IBA; V3: MS medium supplemented with 0.5 mg $L^{-1}$ IAA, 3 mg $L^{-1}$ BAP and 20 g $L^{-1}$ sucrose; V4: MS medium supplemented with 0.18 mg $L^{-1}$ AIA and 2.25 mg $L^{-1}$ BAP with 20 g $L^{-1}$ sucrose).

## CONCLUSIONS

Our paper underlines the first successfully performed studies on *in vitro* *Achillea oxyloba* ssp. *schurii* (Sch.Bip.) Heimerl callus culture besides their antioxidant and antimicrobial potential. Further studies are needed to determine the most efficient *in vitro* culture media composition and parameters for *in vitro* culture optimization. *Achillea oxyloba* ssp. *schurii* (Sch.Bip.) Heimerl is responsive species to *in vitro* culture regarding callus obtaining which is a prerequisite for morphogenetic aspects.

**Acknowledgments**

This paper was supported by project RO1567-IBB06/2020, RO1567-IBB05/2020, and RO1567-IBB03/2020 from the Institute of Biology Bucharest of the Romanian Academy.

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