THE ANTIMICROBIAL AND BIOTECHNOLOGICAL POTENTIAL OF *Ocimum basilicum* L. CORRELATED WITH DEVELOPMENTAL STAGE AND CULTIVAR TYPE

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Abstract. In this study, we considered the following criteria to assess the *Ocimum basilicum* L. (Labiatae) reactivity against the *Staphylococcus aureus* ATCC 25923 bacterial strain: the type of cultivar (Romanian, Italian and Greek basil), the developmental stage of the *in situ* plant material tested (plantlets, plant before flowering period, plant in flowering period), the callus formations differentiated *in vitro*, the interval of reactivity by considering two time marks, 4, respectively 24 hours. The samples were tested for antibacterial activity by determining the number of colony-forming units per millilitre (CFU/mL) in relation to the positive control; serial decimal dilutions were made from each sample. The results showed that both the time interval used to test the reactivity, the type of cultivar and the developmental stage influence the percentage of bacterial reduction. Comparing the data of different regarding the reduction of bacterial colonies, we appreciated that a good reactivity had callus formations obtained *in vitro*. Our obtained results underline the different characteristics of basil varieties in response to bacterial contamination, Italian basil plantlets expressing the best reactivity, followed by callus formations of Romanian basil, then Greek plant before the blooming period and Romanian plant in the blooming stage.

Keywords: *in vitro Ocimum basilicum* L. culture, callus differentiation *in vitro*, *in vitro* antibacterial activity, sweet basil cultivars, pharmacological properties of *Ocimum basilicum* L.

Rezumat. Potențialul antimicrobian la *Ocimum basilicum* L. corelat cu stadiul de dezvoltare și genotipul acestuia. În acest studiu, am luat în considerare următoarele criterii de evaluare a reactivității busuiocului, *Ocimum basilicum* L. (Labiatae) față de tulpina bacteriană *Staphylococcus aureus* ATCC 25923: varietatea de busuioc (românesc, italian, grecesc), stadiul de dezvoltare *in situ* al materialului vegetal testat (plantule, planta înainte și în timpul perioadei de înflorire, formațiunile calusale diferențiate *in vitro*, intervalul de reactivitate raportat la două repere de timp, și anume 4, respectiv 24 de ore. Probele au fost testate cu privire la activitatea antibacteriană prin determinarea numărului de unități formatoare de colonii (CFU/ml) în raport cu martorul; au fost realizate diluții zecimale seriale pentru fiecare probă. Rezultatele au arătat că atât intervalul de timp utilizat pentru a testa reactivitatea, cât și tipul de varietate de busuioc și stadiul de dezvoltare al plantei influențează semnificativ procentul de reducere bacteriană. Comparând datele referitoare la activitatea de reducere bacteriană, apreciem o reactivitate bună a formațiunilor calusale obținute *in vitro*. Rezultatele noastre subliniază caracteristicile diferite ale soiurilor de busuioc testate, ca răspuns la contaminarea bacteriană, plantulele de busuioc italian exprimând cea mai bună reactivitate, urmate de formațiunile calusale de busuioc românesc, busuiocul grecesc înainte de perioada de înflorire și planta de busuioc românesc în perioada de înflorire.

Cuvinte cheie: cultura *in vitro* la specia *Ocimum basilicum* L, diferențierea de calus *in vitro*, activitatea antibacteriană *in vitro*, soiuri de busuioc, proprietăți farmacologice ale speciei *Ocimum basilicum* L.

INTRODUCTION

The plant *Ocimum basilicum* L. belonging to the Lamiaceae family represents an attractive source for both commercial and culinary values in traditional Italian and Thai cuisine. Reticence towards antibiotic use in treating infections with *Staphylococcus aureus*, which reaches a high percentage amongst children and the elderly, has led to the search for new solutions among medicinal plants. This plant raised special attention among species with content of antioxidants. The presence of several essential oils (PURUSHOTHAMAN et al., 2018) in various parts of the plant (root, leaf, etc.) is generally associated with antibacterial properties of the *O. basilicum* L. (KOROCH et al., 2017).

Phytochemical and pharmacological studies point out the immunomodulatory activity of *Ocimum basilicum* (CH MA et al., 2015; LOVETH & LUCKY, 2018) and also antioxidant, antibacterial, antiviral, antifungal, hypoglycemic, hypolipidemic and hepatoprotective actions (RUBAB et al., 2017). A number of identified active constituents including: volatile oils, saponins, coumarins, alkaloids, tannins, anthra-quinones, anthocyanins, flavonoides, diterpenoides, tri-terpenoides, pyredines, pyrolidines, polyphenols, irridoides, quinones, sugars and insect moulting hormones (PADALIA et al., 2017; TSASI et al., 2017) argues for the biological activities of *O. basilicum* L. Concerns about the *in vitro* culture of the species are dated from 1997 (SAHOO et al., 1997) to tour days, as this technique is an alternative to pollution, to the insecticidal attack of the mature plant or to unfavourable weather conditions. BREZEANU & COGĂLNICEANU (2005) presented experimental *in vitro* conditions for callus culture initiation and somatic embryogenesis. Callus extract antimicrobial activity was described first, by SHAFIQUE et al. (2011). Later, SUMAIRA et al. (2017) performed the green synthesis of silver nanoparticles (AgNPs) using *Ocimum basilicum* L. var. *thyrsiflora* leaf derived callus extracts and investigated their antibacterial activity.

Sweet basil callus is a source of natural antioxidants (WONGSEN, 2015). Callus cultures of *Ocimum basilicum* L. showed *in vitro* antioxidant activities and *in vivo* protective effects against UV stress by production of phenylpropanoid metabolites with (NAZIR et al., 2019). In their studies, ILIĆ et al. (2019) highlight the chemical compounds chemotype correlation of some basil cultivars to the environmental conditions and origin of the plant. Studies were conducted also on the vegetative development and ornamental potential of basil cultivars by FRANÇA et al.

(2017). Culture conditions like artificial led lighting have proved to enhance the growth characteristics and phenolic content of *Ocimum basilicum* (BANTIS et al., 2016). Essential oil content and also the cultivation site was reported by TSASI et al. (2017) for five varieties of *Ocimum basilicum* L. from Greece. The antibacterial potential of various parts of *O. basilicum* L. plant has been extensively investigated over the years (SILVA et al., 2016; ALTIKATOGLU et al., 2017; SUMAIRA et al., 2017; TAECHOWISAN et al., 2018; MAJDI, 2020). FLANIGAN & NIEMEYER (2014), KASEM (2017) reported that plant maturity and cultivar play a key role in total phenolic and anthocyanin content. NEGAHBAN et al. (2015) reported the effect of different harvest stages on the quality and quantity of the essential oil of tulsi.

KAKARAPARTHI et al. (2015) presented the antimicrobial activity of the essential oils of two varieties of *Ocimum basilicum* harvested at short time intervals beside the composition of herb and seed oil. ZAREEN et al. (2014) performed a screening of the antibacterial potential of holy basil and Italian basil essential oils. A characterization of essential oil composition in different basil species and pot cultures by a GC - MS method has been performed by MURÁRIKOVÁ et al. (2017). In Romania, this aromatic species was described as early as 1961 by GRINȚESCU (1961) who argued it had various uses in religious ceremonies and traditional Chinese medicine from ancient times. Starting from the mentioned considerations, this paper deals with a comparative antimicrobial study of callus and vegetal material sampled in different developmental stages from the Romanian, Italian and Greek varieties of *O. basilicum* L. A brief description of these intraspecific forms of basil is available in a review of MAKRI & KINTZIOS (2007). Also, our purpose was to test the percentage of bacterial reduction capacity after 4, respectively 24 hours, of callus, plantlets and mature basil plant. The bacterial strain was *Staphylococcus aureus* ATCC 25923.

MATERIAL AND METHODS

Plant material. Research was carried out on three varieties of commercial basil: Romanian, Italian and Greek, for assessing antibacterial activity against fresh bacterial culture of *Staphylococcus aureus* ATCC 25923. The plants were cultivated in May and the vegetal material was harvested from potted plants in different maturity stages, from plantlets to plants in blooming period. The *in vitro* material represented by callus formations was also tested. Special attention was paid to avoid downy mildew for potted plants, following the method of SILVA et al. (2019).

Callus cultures were initiated on MS containing 1, 1.5, and 2 mg/l 2.4 D (2, 4-dichlorophenoxy acetic acid) then were incubated in a growth chamber at 24 °C under 16/8 h photoperiod. Callus sample was sampled for analysis after one month and was subsequently replanted on a fresh medium every month to maintain the collection.

***In vitro* establishment of callus culture**. The initial tissue for callus induction was isolated from 10-12 days old *in vitro* grown seedlings. Plantlets were obtained in vitro from seeds sterilized by carefully washing them under running tap water for 30 min in a gauze-covered jar. The surface decontamination of seeds was performed with 70 % ethanol (30s) and 0.1 % HgCl2 (3 min) rinsed with sterilized deionized water three times. After about 10 – 12 days, plantlets developed from seeds in a Murashige - Skoog (1962) basal medium. The cuttings from *in vitro* regenerated plantlets were inoculated on a Murashige - Skoog (1962) basal medium having 3 % (w/v) sucrose, 0,8 % (w/v) agar and 2 mg/L of 2.4 - dichlorophenoxyacetic acid (2,4 -D) (Figs. 1, 2).

Figure 1. Source for callus induction: regenerated plantlets (4 weeks) induced on MS medium on MS hormone free medium (original).

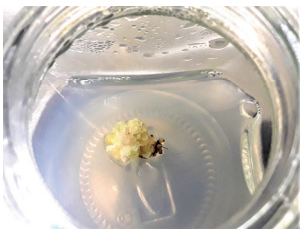

Figure 2. Callus formation: white - yellow callus *in vitro* with 2 mg L^{-1} 2,4 - dichlorophenoxyacetic acid (original).

Evaluation of the antibacterial activity of *Ocimum basilicum* L. vegetable material. A bacterial strain was inoculated on Luria Bertani (LB) culture medium at 37º C, with agitation (150 rpm). In order to observe the effect of the vegetal material on bacterial cells, the vegetable material was weighed and cut into small pieces. These were distributed into a sterile Erlenmeyer flask and 25 mL of Luria-Bertani (LB) liquid growth media and 0.5 mL of liquid inoculum (0.5 McFarland) were added. The inoculated medium was incubated at 37ºC for 24 h with agitation (150 rpm). In parallel, a positive control was prepared, which was treated under the same conditions, but in the absence of the vegetable material. The growth media (LB) had the following composition (g/L): triptone 10, yeast extract 5, sodium chloride 10 and agar 15. The pH of the growth media was adjusted to 7.2 prior to sterilization (121 °C for 20 minutes). After 4h and 24h from the contact of the bacterial inoculum with the vegetable material, the samples were tested for antibacterial activity by determining the number of colony-forming units per millilitre (CFU/mL) in relation to the positive control. In this regard, serial decimal dilutions were made from each sample and 100 µL of them were inoculated by pour plate method into agarized LB growth media. Inoculated plates were incubated at 37 °C for 24h and the experiment was done in duplicate. The inoculum was a fresh

bacterial culture of *Staphylococcus aureus* ATCC 25923. 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. Vegetable material was represented by basil callus obtained *in vitro* (Fig. 2), somatic parts of three potted *Ocimum basilicum* L. cultivars in different stages of maturity (Figs. 3-7).

Figure 3. Italian plantlets (1 week 7 days) (original).

Figure 4. Romanian basil plantlets (2 weeks) (original).

Figure 5. Romanian basil (5 weeks) (original).

Figure 6. Greek basil mature plant (7 weeks) (original).

Figure 7. Plant material of the potted plant (9 weeks): sample 1 = basil inflorescence (0,5 g); sample 2 = basil leaf (1 g).

RESULTS AND DISCUSSION

***In vitro* callus establishment.** *In vitro* callus culture of sweet basil is a method of secondary metabolites production with many useful biological properties including growth inhibition of pathogenic bacteria like *S. aureus* strains. This alternative ways of basil therapeutic potential exploitation was frequently initiated on MS media fortified with 2.4 - D (2, 4-dichlorophenoxy acetic acid) (ABDELRAHMAN et al., 2019). Different concentrations of this phytohormone were used to induce and enhance the callus mass *in vitro*. Hence, ABDELRAHMAN et al. (2019) obtained the maximum growth of basil callus using leaf explants inoculated on an MS medium supplemented with 0.25 mg/L of 2.4 - D. The differentiation and proliferation capacity of the plant callus on growth mediums having 2.4 D (in concentrations of 5 mg/L), has also been tested by LASLO et al. (2014). WONGSEN et al. (2015) obtained the highest callus mass fresh weight, on the medium supplemented with 0.5 mg/l 2.4-D, in 4 weeks. In our experiments, callus developed in a month like a white-yellow cellular mass, with a friable consistency (Fig. 2) adding 2 mg/L 2,4 - D auxin to the MS medium. This result is in agreement with the data of SHARMA et al. (2013, 2014, 2016), who obtained the highest callus mass with 2 mg/L of 2.4 - D supplementation of the MS medium, to *Ocimum canum* Sims, and *Ocimum basilicum* L, from nodal segment explants collected from 6 - 7 months old plants. The results of BHUVANESHWARI et al., (2016) showed that leaf explants are more suitable for callus induction in a concentration of 1 mg/l of 2. 4 - D. Regarding the phytohormonal type, our results are similar with those of BREZEANU & COGĂLNICEANU (2005). The source of explants differed in experimental attempts, but ENKHBILEG et al., (2019) and NAZIR et al., (2019, 2020), also used leaf explants (0.5 cm^2) from *in vitro* developed seedlings of 12 - 14 days, respectively 28 – days old.

Evidence of *in vitro* antibacterial activity of *Ocimum basilicum*. The indigenous basil was screened for antibacterial activity regarding all the important *in situ* developmental stages (plantlets, mature plant, plant with inflorescence) (Figs. 4; 5; 7) and *in vitro* obtained callus (Fig. 2). The impact of basil indigenous *ex vitro* developed samples on bacterial colonies at different dilutions is illustrated in figures (Figs. 8; 9; 10; 11-sample 2; 12). The addition of different types of vegetal samples to the bacterial strain culture significantly affected the number of colony-forming units per millilitre (CFU/mL) in relation to the positive control (Tables 1, 2, 3). The results of these effects are illustrated in figures 9-12. In relation to the positive control, the number of colony-forming units per millilitre (CFU/mL) of *S. aureus* ATCC25923 decreased in the presence of the callus mass obtained *in vitro*.

Figure 8. Bacterial culture after 24h From the inoculation of bacterial strain ATCC 25923, in the presence of indigenous basil callus.

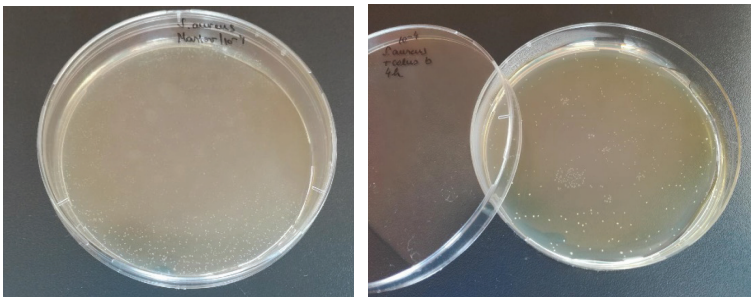

Figure 9. Bacterial colonies at 10^{-4} dilution for *S. aureus* ATCC 25923 strain (positive control - left) and callus (right), 4h after the bacterial contact with basil callus.

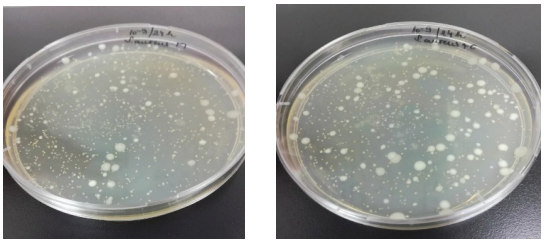

Figure 10. Bacterial strains at 10^{-9} dilution for *S. aureus* ATCC 25923 strain: (positive control - left) and sample (right), 24h after bacterial contact with basil callus.

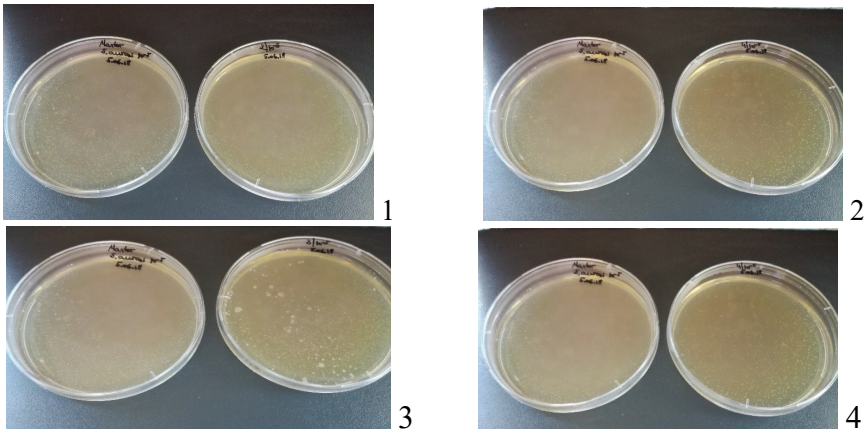

Figure 11 Bacterial colonies at 10^{-5} dilution for *S.aureus* ATCC 25923 strain: (positive control) and samples (1, 2, 3 and 4) 4 h sfter bacterial contact with basil.

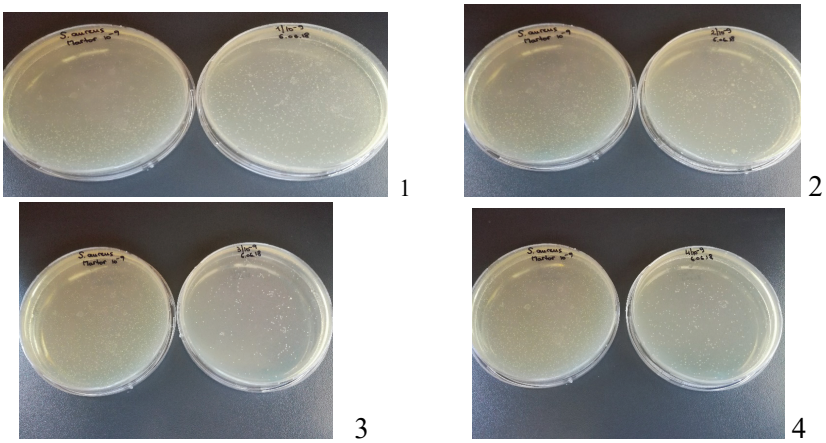

Figure 12. Bacterial strains at 10^{-9} dilution for *S. aureus* ATCC 25923 strain: (positive control - left) and samples (right) (1, 2, 3 and 4), 24h after the contact with bacterial strain.

Table 1. Bacterial activity of callus sample, 4h and 24h after the contact with the bacterial strain.

Sample	T4h	T24h
Callus obtained *in vitro +S.aureus ATCC25923*	$3,5x10^6$ ufc/mL	$6.08x10^{11}$ ufc/mL
Positive control *(S.aureus ATCC25923*)	$1,6\ x10^7$ ufc/mL	$1.16x10^{12}$ ufc/mL
Percentage of bacterial reduction	78,12%	47.58%

Table 2. Antibacterial activity of *Ocimum basilicum* plant material of the potted plant during the preblooming period.

Sample	T4h	Percentage of bacterial reduction 4h	T24h	Percentage of bacterial reduction 24h
1. Italian basil plantlets (7 days)	$3,16x10^7$ ufc/mL	82,3%	$2,2x10^{12}$ ufc/mL	11,3
2.Inland basil plantlets (2 weeks)	$1,78x10^8$ ufc/mL	0,5%	$1,06x10^{12}$ ufc/mL	57,2 %
3. Inland basil plant in the preblooming stage (5 weeks)	$1,72x10^8$ ufc/mL	3,9%	$1,03x10^{12}$ ufc/mL	58,4 %
4. Greek basil plant	$1,43x10^8$ ufc/mL	20,1%	$5,28x10^{11}$ ufc/mL	78,7
Positive control *(S. aureus ATCC25923*)	$1,79\ x10^8$ ufc/mL		$2,48x10^{12}$ ufc/mL	

Table 3. Antibacterial activity of the *Ocimum basilicum* plant material of the potted plant during the blooming period.

	UFC/mL	4h Percentage of bacterial reduction compared to the positive control	UFC/mL	24h Percentage of bacterial reduction compared to the positive control
Positive control	$2.48\ x\ 10^8$		$7\ x\ 10^9$	
Sample 1 (basil inflorescence)	$1.41\ x\ 10^8$	43.14%	$2,5\ x\ 10^9$	64.28%
Sample 2 (basil leaves)	$2.25\ x\ 10^8$	9.27%	$5\ x\ 10^9$	28.57%

The highest percentage of bacterial reduction and the most effective response (in the first 4 hours) was registered by Italian plantlets. Also, in 24 hours, the mature Greek plant registered a high antibacterial activity. Comparing the data of different samples regarding the percentage of bacterial reduction, we appreciated that a prompt response (respectively in the first 4 hours) was recorded by Italian plantlets, callus formations and inflorescence. A later response appeared in the case of the Greek basil plant, the inflorescence of Romanian basil, Romanian basil in the pre blooming stage and the plantlets.

Concerning the plant part used in study, it results that in contrast to the leaf samples of indigenous basil, the inflorescence was most effective (Table 3). The capacity of bacterial reduction can be explained by the different content in phenolics of the samples (MURASHIGE & SKOOG, 1962; SAHOO et al., 1997; MOGHADDAM et al., 2011); this can be modulated and stimulated in future studies by the *in vitro* conditions, like phytohormonal elicitors. Also, we intend to complete the preliminary studies in improving and assessing the content in secondary metabolites of callus formations of Greek and Italian basil, to screen these formations for antibacterial activity and to include new varieties of basil to *in vitro* conditions, as well as establish the influence of the developmental stage of plant and cultivars on antimicrobial activity**.**

According to previous studies, different developmental stages (vegetative stage, floral budding and full flowering stage of *O. sanctum*) affect the essential oils (Eos) composition in terms of quality and quantity and antibacterial activity against standard strains including gram positive bacteria of *S. aureus* (NEGAHBAN et al., 2015; SAHARKHIZ et al., 2015). Our findings also confirm the studies of ERIOTOU et al. (2015) who proved that antimicrobial activity depends on the basil variety as well as the plant part (leaves and flowers). Our results regarding the high antibacterial potential of Italian basil variety are in agreement with the results of ȘTEFAN et al. (2013) who showed that the oils of *O. basilicum* var. *Genovese* growing in Romania exhibited the strongest antibacterial effect against *S. aureus*.

The indigenous basil herb was evaluated for phytochemical screening and antioxidant activity (GIRD et al., 2015; TRETTEL et al., 2018). SILVA et al. (2016) correlates the antibacterial activity against *S. aureus* of leaves components of *Ocimum* with linalool properties. The experiments of TAECHOWISAN et al. (2018) proved the antibacterial property of crude extract and major isolated compounds, linalool and 1,8 - cineole against *S. aureus*.

CONCLUSIONS

An important detail of our experiments and our original contribution in the study field is that we established an interval of time of bacterial reactivity for basil callus obtained *in vitro* (established at 4, respectively 24 h), and that we succeeded in assessing the antimicrobial activity for developmental stages (plantlets, plant in pre -blooming stage, plant in flowering period) for indigenous basil and to test samples of plantlets and leaves for two *Ocimum* cultivars, namely Italian and Greek. These studies were not performed before. The results we obtained underline the different characteristics of basil cultivars in response to bacterial contamination. The antimicrobial activity depends on the *Ocimum* cultivar type and also on the developmental stage.

Our findings argue for potential applications like useful nutritional supplements with antimicrobial therapeutical effects.

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