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IN VITRO CULTURE AND ANTIMICROBIAL ACTIVITY OF *Ocimum basilicum* L. var. 'Spicy globe' AND *Artemisia eriantha* Ten.

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Abstract

In this preliminary screening, we assessed the antibacterial activity of in vitro obtained plant material, namely shoots of Artemisia eriantha (Asteraceae) and callus of Ocimum basilicum L. var. 'Spicy globe', against two bacteria strains, the Gram-positive ones including Staphylococcus aureus ATCC 25923, and Gram-negative, Escherichia coli ATCC 25922 by the plate-counting method. Artemisia sprouted on the Murashige-Skoog medium added with 1.5 mg/L benzylaminopurine and 'Spicy globe' basil developed callus on Murashige - Skook basal medium supplemented with 2.5 mg/L naphtylacetic acid (NAA). The method of using small pieces of vegetal material inoculated in Erlenmeyer flasks on liquid Luria-Bertani medium added with standardized microbial cell suspension was not effective against the pathogenic bacteria in the case of the Artemisia species, but the same method was efficient for basil cultivar 'Spicy globe'.

Key words*: in vitro Ocimum basilicum L. culture, Artemisia in vitro culture, the antioxidant activity of basil, the antibacterial and antioxidant activity of Artemisia.*

INTRODUCTION

It is a high commercial demand regarding medicinal plants. Plant tissue culture technique prevents the inconvenience of susceptibility to infestation with bacteria, fungi, and insects, which can influence the properties of the preparations by growing them to a large extent in controlled conditions in a limited time (Monga et al., 2017). Also, natural extinction due to the different natural and anthropogenic factors can be avoided by tissue culture of special genotypes and chemotypes. By cloning these, all the cells will have the same genetic heritage.

Introducing in *in vitro* culture and antimicrobial activity of cultivar basil 'Spicy globe' (*Ocimum basilicum*)

At least 18 different cultivars of *O. basilicum* are mentioned by the Herb Society of America (Majdi et al., 2020), intraspecific variability being a specific characteristic for basil related to certain parameters (Attia et al., 2011). A wide range of studies have been conducted on the biotechnological potential of basil, correlated with cultivar type and developmental stage (Voicu et al., 2020), morphogenetic response *in vitro* from different basil lines (Dănăilă-Guidea et al., 2020), selection, and micropropagation of germplasm lines with high content in antioxidant phytohormones as melatonin and serotonin (Ferrarezi & Bailey, 2019) rapid multiplication *in vitro* by multiple shoot formation (Monga et al., 2014; Pattnaik et al., 1996).

'Spicy globe' is an intraspecific form of Bush or Greek Basil (common name) or *Ocimum basilicum minimum* (Latin name), after Makri and Kintzios (2007), beside 'Fine Green' and 'Green Bouquet', adapted to tropical warm conditions (Simon et al., 1999). On the other hand, Attia et al. (2011) mention the variety characterization of Simon (1999) as a popular hybrid between *O. americanum* and *O. basilicum*, with a compact little bush densely covered with small leaves containing aroma compounds linalool, 1.8 - cineole and methylchavicol. Essential oils containing linalool and eugenol (Verma et al., 2011; Taechowisan et al., 2018) increase the antibacterial potential of basil (Silva et al., 2016).

The significant antioxidant effect of linalool contained in *Ocimum basilicum* L. increases antibacterial activity, having a synergistic activity with the existing standard antibiotics

(Saharkhiz et al., 2015). Also, the eugenol component expresses strong antibacterial activity against *S. aureus* (Taechowisan et al., 2018; Silva et al., 2016; Saharkhiz et al., 2015; Gochev & Girova, 2014). Previous studies revealed that the response to bacterial contamination against certain strains depends on the cultivar type, the developmental stage *in vitro* and *ex situ*, the differentiation process of the vegetal tissue *in vitro* (Voicu et al., 2020), the phytochemical composition of the basil cultivars (Majdi et al., 2020), free radical scavenging activities against Gram-positive relative to Gram-negative bacteria (Devi et al., 2013). Species of basil has a differential reaction and different potential in reduction bacterial activity. *Ocimum gratissimum* has antibacterial activity against *S. aureus* in a 0.1% minimum inhibitory concentration (Voicu et al., 2020). Gram-negative bacteria strains as *E. coli* are sensitive to essential oil components of *Ocimum basilicum* L. (Eriotou et al., 2015).
Further studies intend to develop new biotechnologies to increase *in vitro* biomass to different basil cultivars and to enhance antimicrobial activity, to combat antibiotic resistance (Nabrdalik & Grata, 2016) based on essential oils content modulation using different recipes.

In vitro micropropagation and antimicrobial activity of *Artemisia eriantha* (Asteraceae)

Artemisia eriantha Ten. is an alpine phytotaxon with protected status at the national and European level with rich content in terpenoid compounds as sesquiterpenes that increase with the age of plant (Predoi et al., 2018). *Artemisia eriantha* Ten. presents in natural areal a rarely and new encountered process of *ex vitro* micropropagation namely fasciation (Reale et al., 2014). However, *in vitro* micropropagation of plants presents a series of benefits. Previous studies established direct shoot regeneration from nodal segments of the plant to *Artemisia eriantha* Ten. (Pace et al., 2020), *Artemisia vulgaris* L. (Voicu, 2017), and *Artemisia annua* (Jogam et al., 2020; Zayova et al., 2018). Genotypes of *Artemisia* with a high potential of producing useful substances for human health as artimisinin are of great interest for clonal propagation *in vitro* (Zayova et al., 2020). An optimum formula for *Artemisia* species shoots induction is 0.5 mg/L BAP and 0.25 mg/L kinetin and repeated subculturing for rapid multiplication of shoots with 1 mg/L BAP and 1 mg/L kinetin and 0,1 NAA (Wetzstein et al., 2018). *In vitro* development of some *Artemisia* species, as *Artemisia absinthium* seed germination, root and shoot length, was effectively improved by modern techniques as metallic nanoparticles application (Shekhawat et al., 2015). The *Artemisia* species present a wide range of biological activities due to the chemical compound as phenolic acids (Hussain et al., 2017). The biological activities of *Artemisia* species and biochemical compounds are extensively studied ((Hussain et al., 2017). The major compounds of the *A. eriantha* wild plant identified by Milosavljevic et al. (2001) are thujone. *In vitro* shoots content in polyphenols and flavonoids of *A. eriantha* is reduced compared with the wild plants (Pace et al., 2020). Using n-hexane extract of *Artemisia parviflora* leaves, Ahameethunisa and Hopper (2012) (Milosavljevic et al., 2001) inhibited the growth of *Escherichia coli*. Combined ethanol extracts of native *A. oliveriana* and *A. aucheri* from Iran land have an inhibitory effect on *S. aureus* ATCC 33591 (Ahameethumisa & Hoopper, 2012). Mainly native *Artemisia* species were determined regarding their antimicrobial activity. Related to in vitro obtaining *Artemisia* regenerants, many studies on *Artemisia* species from natural habitats highlight their antimicrobial activity. Water, ethanol, and methanol extracts of the *A. iwayomogi* and *A. princeps* were evaluated regarding antimicrobial activities (Baghini et al., 2018). *Artemisia rupestris* flavonoids chrysosplenetin, penduletin, and chrysoeriol exhibited synergistic activity in association with norfloxacin against *Staphylococcus aureus* 1199B strain (Pak & Jun-Hyun, 2019).
Based on the main author, this is the first report on *in vitro* reactivity and antimicrobial potential of 'Spicy globe' basil variety and liqueur genepi *Artemisia eriantha*.

MATERIALS AND METHODS

Plant material

'Spicy globe' basil seeds purchased from the store www.semintelegumeflori.ro were placed in Petri dishes containing wet cotton-wool

covered with paper filter discs; after germination, the plantlets were sterilized and cultured *in vitro* to the laminar flow bench on the culture media MS + 2.4 D (2 mg/L) for callus induction (Voicu et al., 2020) and subcultured on supplemented media Murashige - Skoog with 2.5 mg/L NAA (Lan et al., 2021). Seeds of the mature plant *Artemisia eriantha* Ten. harvested by Nicoara R., researcher to the Department of Ecology of Bucharest Institute of Biology, to the end of August 2020 from the alpine area of the Bucegi mountains, were placed for germination in Petri dishes on filter paper wet discs covering a cotton-wool layer. For *Artemisia eriantha* Ten. seeds, the germination process was stimulated by using alternatively air conditioning flow of 15°C/20°C at an interval of 12 hours. The plantlets served as an inoculum source for *in vitro* multiplication. The sterilization procedure used similar steps to the method tested in the previous experiments (Pace et al., 2020). For the *in vitro* shoot induction and multiplication of *Artemisia* sp., we choosed the simplest and optimum variant of Murashige-Skoog culture medium improved with 1.5 mg/L benzyl-aminopurine, after the method of Jogam et al. (2020) (Voicu, 2017).

Test microorganisms

In this study, we investigated the antibacterial activities of *Ocimum basilicum* callus and *Artemisia eriantha* shoots on the Gram-positive and Gram-negative bacteria. *In vitro* antibacterial studies were carried out on two bacteria strains, the Gram-positive ones including *Staphylococcus aureus* ATCC 25923, and Gram-negative ones, *Escherichia coli* ATCC 25922. Both strains were grown aerobically at 37°C in Luria Bertani medium culture with the following composition (g/L): tryptone 10, yeast extract 5, sodium chloride 10, and agar 15.

Determination of antibacterial activity

In vitro antibacterial activity was assessed by the plate-counting method. 1 g of vegetable material was weighed and cut into small pieces under sterile conditions 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 cell suspension (10^8 CFU/mL). The inoculated flasks were incubated at 37ºC in aerobic conditions, with stirring. Positive control was prepared in parallel, differing only by the absence of the vegetable material. After 4 h and 24 h from the contact, the number of surviving bacteria in agar plates was counted after 24 hours of incubation at 37ºC and was expressed as CFU/mL. The percentage of bacterial reduction was calculated using as reference the control treatment. All experiments were prepared in triplicate to ensure data reproducibility.

RESULTS AND DISCUSSIONS

Initiation of *in vitro* culture and antimicrobial activity of vegetal material

To avoid supplementary contamination of the explants, we obtained the source of explants as plantlets in Petri dishes on wet filter discs. The period of germination varied considerably between the two species, regarding the fact that the germination of *A. eriantha* L. lasted 17-25 days in September and for the 'Spicy globe', 6-7 days, in thermal comfort. The rate of germination was 80 % for 'Spicy globe' basil and 40 % for *A. eriantha*.

***In vitro* culture of *Ocimum basilicum* var. 'Spicy globe'**

Callus cultures of 'Spicy globe' basil were initiated on MS added with 2.4 - D auxins, as in previous studies, to *Ocimum basilicum* L. (Voicu et al., 2020) and further subcultured on 2.5 mg/L-naftylacetic acid (NAA) supplemented medium (Lan et al., 2021). Also, a considerable yield of callus was obtained by Nazir et al. (2020) with the addition of 5 mg/L BAP to 1 mg/L NAA added to basal MS medium. Spicy globe cultivar maintains its capacity to release volatile compounds *in vitro*, as *ex vitro*.

***In vitro* shoot induction to *Artemisia eriantha* L.**

We succeed to obtain plantlets from mature seeds of the *Artemisia eriantha* plant on wet paper filter discs placed on Petri dishes during 17-25 days in September and to initiate *in vitro* culture of *Artemisia eriantha* by subculturing fragments of plantlets. The MS culture medium supplemented with 1.5 mg/L BAP responded

well to *in vitro* shoot initiation and propagation; we obtained similar results with these of Jogam et al. (2020) (Voicu, 2017). In our previous experiments (Pace et al., 2020), we used nodal segments as inoculum sources, on a wide variety of optimised culture media recipes, associating at least two types of plant growth regulators (PGRs), namely BAP (benzylaminopurine), GA3 (gibberellic acid), and TDZ (thidiazuron) in different proportions.

Antimicrobial activity of *Ocimum basilicum* var. 'Spicy globe' and *Artemisia eriantha*

In this study, we examined the antimicrobial activity of *Ocimum basilicum* callus and *Artemisia eriantha* shoots against pathogenic bacteria to evaluate their potential as antibacterial agents. The results of the experiment were depicted in Figures 1 and 2.

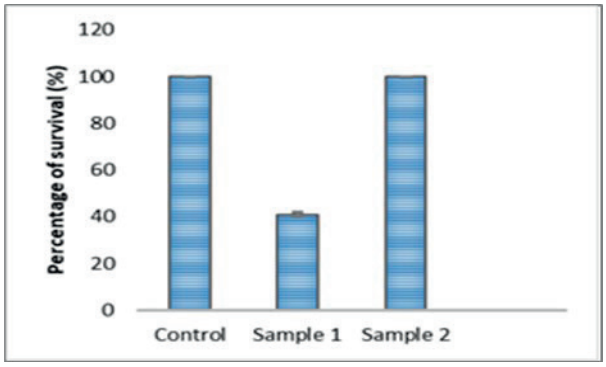

Figure 1. Percentage of survival of *S. aureus* bacterium colonies after 24 hours of incubation with different samples of plant material

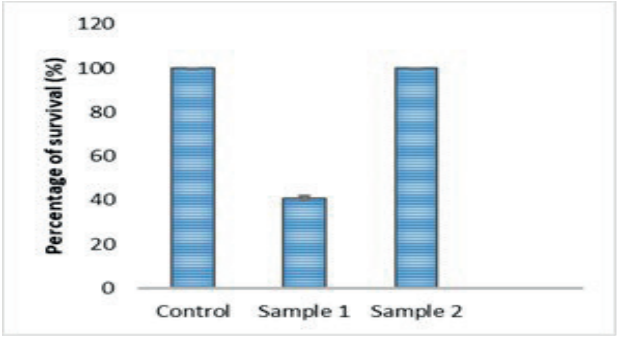

Figure 2. Percentage of survival of *E. coli* bacterium colonies after 24 hours of incubation with different samples of plant material

After 24 hours of contact of bacterial cells with *Artemisia eriantha* shoots, no growth inhibition was observed compared to the control sample. In contrast, good efficacy was found for sample 1 of vegetable material, resulting in a bacterial reduction after 4 and 24 hours of incubation with *E. coli* cells, of 20%, and 66.14%, respectively. A similar effect was also observed for *S. aureus* cells under the same treatment conditions. Thus, a percentage of bacterial reduction of 31% was obtained after 4 hours of contact with the sample of plant material. When a longer incubation was applied, the percentage of cells reduction increased to 59.23%.

Sample 1. *Ocimum basilicum* L. var 'Spicy globe' callus obtained on MS medium added with 2.5 mg/L naphthylacetic acid (NAA)

Sample 2. Microshoots primordia from plantlet fragments of *Artemisia eriantha* differentiated on modified MS medium added with 1.5 mg/L benzylaminopurine (BAP)

CONCLUSIONS

Artemisia eriantha can be micropropagated *in vitro* starting from seeds of the donor plant as well as from nodal segments, although the seed germination process is triggered with difficulty; generated plantlets are a source of healthy, clean material, with young tissues, for *in vitro* multiplication. The antimicrobial activity of *Artemisia eriantha* micro shoots in the initial stage of development on Murashige - Skoog medium enriched with 1.5 mg/L benzylaminopurine is not significant.

Further studies are needed to screen the *Artemisia eriantha* reactivity of *in vitro* formations with repeated subcultures, in order to accumulate the compounds with antioxidant or antibacterial properties, growing them on different variants of enriched culture media. *In vitro* differentiated spicy-globe basil formations release volatile compounds in culture vessels and are reactive to bacterial strains.

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