Particularities of *in vitro* culture of *Ocimum* species including new varieties

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Abstract. The aim of the study was to review the recent scientific literature on morphogenetic potential of *Ocimum basilicum in vitro* and to introduce in *in vitro* culture new varieties as spicy globe beside common basil and scented cinnamon. Different *in vitro* culture systems were presented highlighting the most efficient recipes. No reports are available regarding *in vitro* culture and callus obtaining of spicy globe basil cultivar, our study being the first one on this research field.
Key Words: culture media, germination, micropropagation, tissue culture.

Introduction. The demand of a rich content in secondary metabolites from medicinal plants makes necessary the application of *in vitro* tissue cultures for micropropagation purpose (Wawrosch & Zotchev 2021). In this context *in vitro* tissue culture is a challenge and opportunity for every plant species, mainly those with medicinal and conservative values. This desiderate implies a laborious protocol (Espinosa-Leal et al 2018). Basil (*Ocimum basilicum*) in Lamiaceae family stands out through bioactive complex compounds with antibacterial, antioxidant and anti-inflamatory adjuvant properties and source of functional food (Carović-Stanko et al 2016). Every basil variety either *O. basilicum* var. *basilicum* cv. *genovese* or *O. basilicum* var. *purpurascens* cv. dark opal or *Ocimum x citriodorum* (Paparozzi et al 2021) need in *in vitro* particular conditions related to media composition, as source of nitrogen or percentage of macronutrients or micronutrients. Strength media, namely 2 MS (Murashige-Skoog), MS, ½ MS, ¼ MS and different PGR (plant growth regulators) proportions influence obviously the chemical composition of the aromatic fraction of *O. basilicum* plantlets. Therefore, media 2MS and ¼ MS increase methyleugenol content in basil *in vitro* cultures (Monfort et al 2018). Full strength of culture medium salts (100%) is preferred for *in vitro* seedlings than 0, 25, 50 and 70% concentrations. Also, a double concentration of sucrose of usually used (30 g L^{-1}) determine osmotic stress. Activated carbon in the medium increase the concentration of copper and zinc in the root but reduce the callus formation (Trettel et al 2018). Micronutrients influence the crop in field cultures as *in vitro*. Therefore, zinc is a micronutrient that enhances the field production of basil regarding biomass, nutrient acquisition and also attenuates the negative impact of saline stress on basil (Tolay 2021). Heavy metals as copper determine an allergenic activity of basil, by the accumulations of specific proteins related to transpiration and photosynthetic processes (Georgiadou et al 2018). The plant productivity and quality require phytohormones as brassisteroids that action both in normal as in stress conditions (Li et al 2016; Manghwar et al 2022).

Micropropagation *in vitro* implies three main pathways (Kasem 2017), namely (1) direct micropropagation, (2) indirect micropropagation via callus cells, and (3) somatic embryogenesis pathway, through the conversion of the somatic cells into somatic embryo similar with the zygote. For different basil cultivars with distinct chemical profiles and different reactivity to different culture media composition a series of protocols have been standardized in order to obtain, by different *in vitro* tissue culture types, correlated with the effect of growth regulators, phytochemically consistent crops. Initiation of the *in vitro* culture starts from healthy inoculli as axillary vegetative buds prelevated from basil plants and cultured *in vitro* in sterile conditions on phytohormones supplemented media,

before undergoing the preinoculation procedures that assure the asepsis. Average number of shoots, number of internodes, shoot length, the percentage of rooting, number of roots and root length, are parameters quantified in basil tissue culture in relation with the different concentration of corresponding plant growth regulators added to the Murashige and Skoog medium, as benzyl adenine (BA), naphtyl acetic acid (NAA), kinetin (Kn) or IBA (indole-3-butyric acid). Sterilization of explants in tissue culture is an important stage of the protocol. It is well known that exposure time for sterilisation is a critical parameter for explants decontamination. The bacteria or virus elimination involves adverse effects on *in vitro* tissue cultures (Magyar-Tabori et al 2021) but AgNPs (BNS) (biosynthesized nanosilver) are not limited by time exposure and are a very effective antimicrobial agent for surface decontaminant, without adverse effects on explant viability. Sensibility of the basil plant implies many efforts to find the adequate chemical agent for avoiding the contaminants. Among effective chemical agents used for sterilisation are 2.5% NaOCl (Ekmekci & Aasim 2014), biosynthesized nanosilver (Adebomojo & Abdulrahman 2020), etc. Also, the procedure of sterilising basil seeds is difficult because of the mucillage sheat developed to the contact with the water. An alternative way to obtain sterile plantlets as explants source, is the growing of the seeds in pots covered with a transparent perforated plastic sheet or more efficiently is the germination on wet filter paper discs covering a layer of cotton wool in Petri dishes. A high shoot regeneration frequency is the target for a succesfull protocol of *in vitro* micropropagation. In this context, the initiation stage plays an important role to induce the morphogenetic way by modulating the phytohormone types and appropriate concentrations. Multiple shoot proliferation *in vitro* potential can be tested by minimal use of hormone concentrations as (0.1 mg L^{-1}; 1 mg L^{-1}) NAA or (0.1 mg L^{-1}; 1 mg L^{-1}) indole acetic acid IAA (Jamal et al 2016). A lot of adjuvants are used for *in vitro* tissue culture even fungal elicitors or beneficial microorganisms (Helepciuc et al 2014, 2019). Related to organic substances, we can use glycerol as a substitute of sucrose in *in vitro* culture media recipes (Banciu et al 2016). Basil seeds have a good germination potential and differentiation capacity *in vitro* on different media variants as halved MS culture media, halved media added with active coal (3 g L^{-1}) or assoctiation between 0.5 mg L^{-1} IAA and 0.5 mg L^{-1} BAP (Laslo et al 2014). Among basil cultivars, 'Grecco a Palla' has a high germination speed index and seed germination percentage *ex vitro* (França et al 2017). Maximum number of shoot per explant is a main objective for clonal propagation of plant species and cultivars. To accomplish this objective, reproducible protocols were developed. Establishment of cultures, shoot proliferation and multiplication, indirect shoot regeneration, direct shoot development from explants, rooting and hardening, indirect root formation, direct root formation, additional factor affecting micropropagation were reviewed to a range of basil varieties and cultivars, the most species studies being sweet basil (*O. basilicum*) and holy basil (*Ocimum sanctum* (Tulsi) (Gulati et al 2015). Plant developmental processes implies six classes of phytohormones (Curaba et al 2014). Interacting action between auxins and cytokinins determines either rooting (higher auxins), sprouting (cytokinins higher concentrations) or callusing (both auxins and cytokinins are moderated), related to their relative concentrations (Phillips & Garda 2019).

Indirect micropropagation of basil via callus cells. Callus cultures technologies are valuable means to propagate endangered ornamental or medicinal plants and offer an alternative to modulate the content in active substances of plant. Poliphenolic content of callus cultures is increased in contrast to the in field plants as *Achillea oxyloba* ssp. *schurii* (Sch.Bip) Heimerl (Asteraceae) (Voicu et al 2020a). Characteristics like secondary metabolites content and resistance to insecticidal attack are amplified by the technology of transgenic callus. In this context, Efferth (2019) mentions that transgenic callus contains more rutin than wild-type calli, and exposes a significant toxicity towards *Spodoptera litura* and *Helicoverpa armigera* larvae. Shoots can be regenerated from transformed calli; also, callus cultures can be utilized for multiple shoot formation and mass propagation (Kachhap et al 2018). The capacity of regeneration of the callus is obvious by the embryonal buttons or embryos developed on the surface in three weeks,

after Roşan & Agud (2017). Maximum fresh/dry weight of the callus cultures are correlated with basil cultivar type, as *O. basilicum, O. sanctum* and *O. gratissimum* and an adequate elicitor treatment (Mathew & Sankar 2014). Callus cultures can be induced by different methodologies and different *O. basilicum* explants as from leaves to nodal stems, stem segments or root fragments. A lot of protocols have been developed and optimized. Usually, highest callus induction is achieved using 2,4-dichlorophenoxy acetic acid (2.4-D) (Voicu et al 2020b). Sharma et al (2014) obtained a percentage of 94.44% callusing with 2 mg L^{-1} added to the culture medium. Kachhap et al (2018) obtained maximum callusing to *O. sanctum* in MS medium supplemented with 0.1 mg L^{-1} of 2,4-D. Higher concentrations of 2,4-D increase total flavonoid and tannin content althought callus growth is inhibited. Nazir et al (2019, 2020) optimized the protocol of callus formation using 0.5 cm^2 leaves prelevated from 28 days-old plantlets (*in vitro* - derived) cultured on MS media additionally supplied with NAA (2.5 mg L^{-1}). According to Sumaira et al (2017), callus induction can be performed on MS media with 9.0 mM of TDZ, NAA and 2,4-D to var. *thyrsiflora* of *O. basilicum*. Different melatonin concentrations have been used to optimize the callus content by repeated subcultures of the obtained callus in association with TDZ (9.0 mM). 9.0 mM of TDZ induce the highest phytochemical content in callus cultures. Combinations of melatonin and TDZ are more propitious than TDZ alone for the synthesized of AgNPs through this way improving their antimicrobial capacity. Also, 9.0 mM of TDZ combination with 15 mM melatonin enhance phenolic and flavonoid content to 44.9 mg L^{-1} DW and 31.5 mg L^{-1} DW. The obtained callus was used for green synthesis of silver nanoparticles (AgNPs). AgNPs (BNS) (biosynthesized nanosilver) have stimulating action on formation of callus (Adebomojo & Abdulrahman 2020) using the concentrations of BNS of 10, 50 and 100 mg L^{-1}. The ration of 5:1 mg L^{-1} BAP:NAA yields the maximum biomass accumulation (fresh weight (FW): 190 g L^{-1} and dry weight (DW): 13.05 g L^{-1}) as well as enhance phenolic (346.08 mg L^{-1}) production (Nazir et al 2020) to *thai* basil variety. Concentrations of 0.80-2.40 mg L^{-1} TDZ with or without 0.10 mg L^{-1} IBA associating 1.0 mg L^{-1} PVP and 3.0 g L^{-1} activated charcoal determine a maximum percent of callus induction (Ekmekci & Aasim 2014) starting from 12-14 days old *in vitro* grown seedlings.

In the range of tisuue *in vitro* regeneration system, electrical modulation plays a key role (Whited & Levin 2019). So, the rate growth of the callus can be stimulated by a week electric curent applied constantly at values of 1-2 μA (Cogalniceanu 2006). The experimental data of Enkhbileg et al (2019) regarding the secondary shoot regeneration revealed that the best response is induced with 5.0 mg L^{-1} TDZ (thidiazuron) or 1.5 mg L^{-1} BAP (6-benzylaminopurine) on all types of explants except the roots and also callus can be obtained with all cytokinins on all types of explants. By direct and indirect micropropagation, different rate of multiplication response and different percentage were obtained to *O. basilicum* cultivars. An optimal concentration of 6.97 μM Kn added to MS medium allow 100% percentage of germination and multiple shoots induction (Gayatri et al 2014). For secondary shoot regeneration it is necessary a 5.0 mg L^{-1} TDZ (thidiazuron) supplementration of the culture medium or 1.5 mg L^{-1} BAP (6-benzylaminopurine) on all types of explants except the roots; callus can be obtained with all cytokinins on all types of explants (Enkhbileg et al 2019). Repeated subsequent culture of *in vitro* proliferated shoots is a key of the success to multiplication. Therefore, in about 4-5 months, by this technique, it can be obtained from a single nodal segment explant, about 25-30 shoots, induced by 1.0 mg L^{-1} of 6-benzylaminopurine (BA) with 0.5 mg L^{-1} IAA supplemented MS medium (Sharma et al 2014) to *O. sanctum* in a 82% percentage. MS medium added with 2.40 mg L^{-1} TDZ - 0.10 mg L^{-1} IBA determine the maximum number of shoots (3.58) starting from young seedlings and it seems that hypocotyl induce high frequency of shoots (5.17 shoots per explant) with 2.0 mg L^{-1} TDZ concentration (Ekmekci & Aasim 2014).

The present study was conducted in order to evaluate the *in vitro* morphogenetic potential of a new introduced *in vitro* basil variety, namely spicy-globe, beside common basil and cinnamon basil, depending on cytokinin supplemented culture medium and on medium culture composition.

Material and Method. In the recent years a great number of *in vitro* methodologies have been performed in order to assess the optimal concentration of plant growth factors that trigger the best respose of basil varieties explants. We reviewed a great number of *in vitro* methodologies from the recent years in order to assess the optimal concentration of plant growth factors that trigger the best respose of basil varieties explants (Table 1).

Table 1

Comparative methodologies of *Ocimum* species and varieties *in vitro* culture

No.	*Basil cultivar*	*Reference*	*Optimal phytoregulators concentrations added to MS in vitro culture medium*	*In vitro best response of explants*
1	'Alfavaca Green'	Trettel et al (2020)	0.05 mg L^{-1} NAA and 0.1 mg L^{-1} BAP	Plants with greater numbers of leaves
2	*Ocimum basilicum* 'Genovese' (basil)	Trettel et al (2019)	0.1 mg L^{-1} BAP; 0.4 mg L^{-1} BAP and 0.2 mg L^{-1} NAA	Shoot development
3	*O. basilicum*	Lazarevic et al (2020)	0.25 mg L^{-1} BAP and 0.5 mg L^{-1} GA 3	Development of multiple shoots
5	*Ocimum sanctum*	Kachhap et al (2018)	0.1 mg L^{-1} 2,4-D	Maximum callusing
		Nazir et al (2019)	2.5 mg L^{-1} NAA	Callus
6	*Ocimum basilicum* var. thyrsiflora	Sumaira et al (2017)	9.0 mM TDZ	Highest phytochemical content in callus cultures
		Monfort et al (2018)	0.5+2 mg L^{-1} NAA and BAP; 0.5 mg L^{-1} BAP	Maximum callusing percentage

The experiments in this study were achieved during September 2018 – March 2019. Plant material was represented by seeds (purchased from *O.basilicum* plants grown in churchyard or purchased from store (cinnamon basil and spicy - globe basil) (Figure 1).

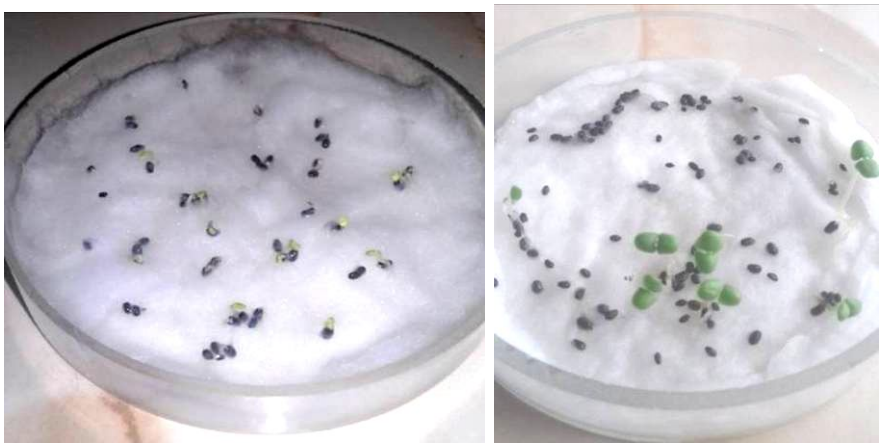

Figure 1. First attempts to test basil germination on moisted cotton-wool in Petri dishes; a slower germination is obvious to cinnamon basil seeds (left side).

For source inoculum we sowed seeds on filter paper discs in order to obtain plantlets as a source of fresh tissue in covered Petri dishes, placed in a warm chamber on the window sill. Another procedure consisted in washing seeds in a series of agents as distilled water, ethanol 70% and dichloroisocianuric acid. Inoculation stage consisted in culture media

distribution in Petri dishes or jars of medium capacity (0.25 mL) or Erlenmeyer dishes and inoculation in a laboratory chamber with sterile condition assured by laminar flow hood or clean benche and inoculation in Murashige-Skoog media variants supplemented with 1 mg L^{-1} of benzylaminopurine cytokinin and 0.5 mg L^{-1} of indolil butiric acid auxin. The inoculated dishes were placed in growth chamber with adecvate environmental conditions. Basal medium of Murashige-Skoog and 2,4-D supplemented medium was tested for common basil; 0.1-1 mg L^{-1} concentrations of BAP in combination with 0.5 mg L^{-1} IBA were tested for inducing the morphogenetic events to the varieties spicy-globe and cinnamon.

Results. The common basil has a good reactivity on *in vitro* culture MS medium, even on basal Murashige-Skoog medium; the 2,4 D supplemented MS variant, generated callus formations to common basil, as in the previous experiments (Voicu et al 2020b); from the 0.1-1 mg L^{-1} benzylaminopurine concentrations tested, 1 mg L^{-1} BAP and 0.5 mg L^{-1} IBA variant induced best morphogenetic response for spicy-globe basil and cinnamon (Figure 4, Figure 5). In the preliminary studies, we tested the germination capacity of basil seeds, capacity to differentiate callus formations and antimicrobian activity (Voicu et al 2020a). In this study we realised a comparation of *in vitro* potential developement of two *O. basilicum* cultivars, namely spicy-globe and cinnamon. Our experiments results revealed that Murashige-Skoog medium supplemented with 1 mg L^{-1} of benzylaminopurine and 0.5 mg L^{-1} of indolil butiric acid regenerated the *O. basilicum* cultivars spicy-globe and cinnamon in about one month, to the beginning of the spring. Cinnamon seeds had a lower potential of germination on wet filter paper discs comparing to spicy-globe (Figure 1). Basil seeds pass a different morphogenetic features until maturity (Figure 2). Basil seeds are reactive to *in vitro* culture; fragments of plantlets can differentiate callus adding phytohormones as 2,4-D (Figure 3). In *in vitro* culture, the phenotype of cinnamon basil differentiated a shorter length and more shoots in contrast to the taller phenotype of spicy -globe (Figures 4 and 5); also, at the base of regenerants spherical callus formations are obvious highlighting that the phytohormonal formula is not the optimal and these species are amenable to callus cultures type in this example.

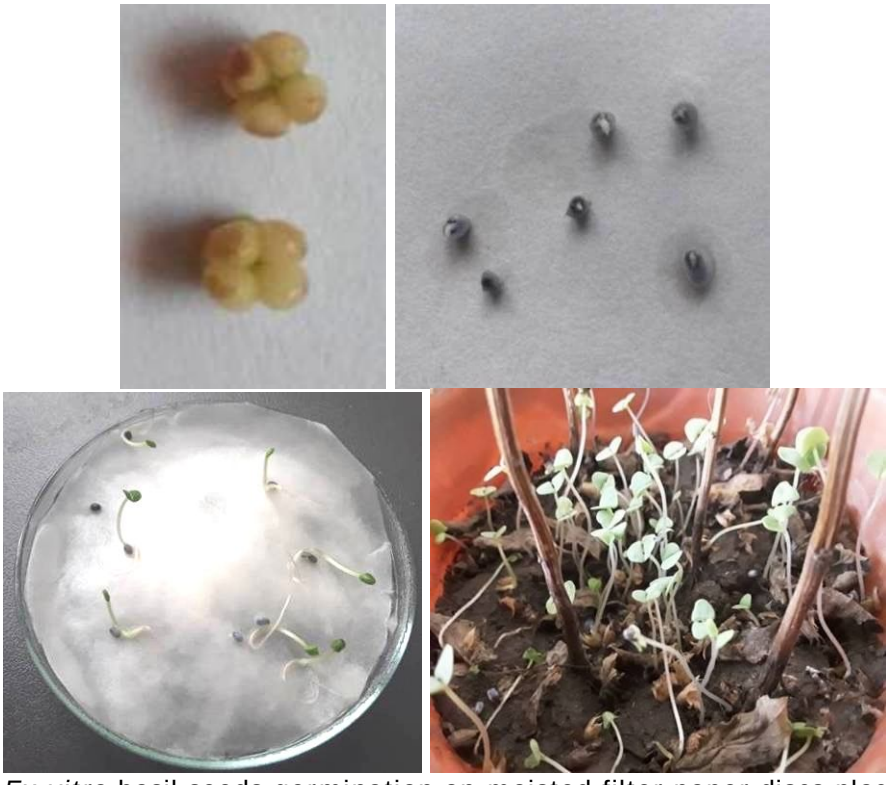

Figure 2. *Ex vitro* basil seeds germination on moisted filter paper discs placed over a cooton-wool wet layer in a Petri dish lead; basil plantlets regenerated on a soil pot.

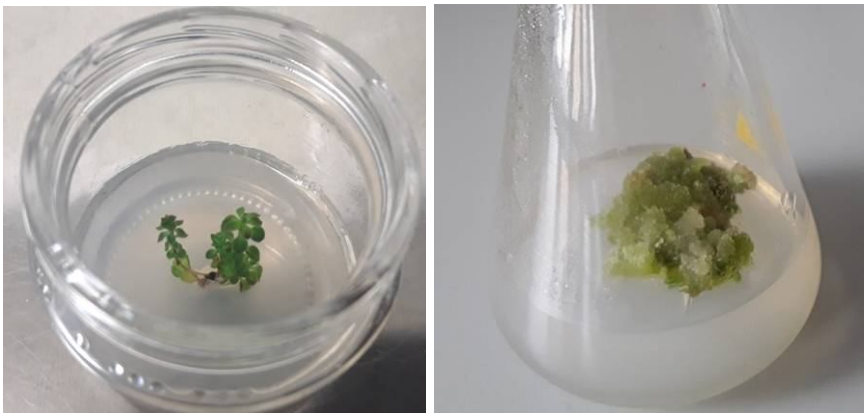

Figure 3. *In vitro* regenerated basil on MS medium from seeds from the mature basil plant seeds. Callus basil developed on 1 mg L^{-1} 2,4-D suppemented Murashige-Skoog culture medium from plantlets fragments.

Figure 4. Spicy globe basil *in vitro* regenerants growing on Murashige-Skoog basal medium added with 1 mg L^{-1} BAP and 0.5 mg L^{-1} IBA.

Figure 5. Cinnamon basil *in vitro* regenerants on 1 mg L^{-1} BAP and 0.5 mg L^{-1} IBA supplemented Murashige-Skoog culture medium.

Discussion. Clonal multiplication of basil starts from shoot buds, nodal explants or axillary buds or seedlings plantlets regenerated from seeds previously on wet filter paper discs (Shahzad et al 2012; Leelavathi & Kuppan 2013; Gaddaguti et al 2015). Growth regulators (auxins and cytokinins) applied in different doses influence *in vitro* organogenesis and growth to all the basil species and cultivars. The growth regulators are correlated with genes of stretching process (auxins) and cell proliferation (cytokinin). Cytokinins are needed to increase the number and length of shoots and the length of

roots (Trettel et al 2019). Low concentrations of NAA (0.05 mg L^{-1}) and 0.1 mg L^{-1} of BAP or kinetin optimise and improve Alfavaca Green cultivar development *in vitro* (Trettel et al 2020). On the other hand, progessively increasing concentrations of naphthaleneacetic acid and benzyl aminopurine (0.6 mg L^{-1} and 0.3 mg L^{-1}) or 0.2 mg L^{-1} benzyl aminopurine (BAP) increased fresh and dry masses of roots (Trettel et al 2019) to 'genovese' basil.

Our results showed that the germination process needs a longer period of time for cinnamon cultivar on filter paper discs than the germination process of spicy globe basil, but this inconvenient was removed by *in vitro* culture.

Conclusions. *In vitro* previous methodologies to regenerate *Ocimum basilicum* are amenable to various characteristics, the most efficiently in stimulating a rapid response of multiple shootregeneration and accumulation of secondary metabolites being: benzylaminopurine, thidiazuron, alone or associated with naphtylacetic acid or gibberellic acid; also predominant elicitors used with good results are yeast extract, methyljasmonate, melatonin, and salicilic acid. Our preliminary results revealed that the germination process takes a longer period of time to cinnamon cultivar on filter paper discs than the germination process of spicy globe basil, but this inconvenient is removed by *in vitro* culture. The potential to regenerate and multiplicate *in vitro* basil cultivars from hypocotyl segments is considerable.

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Conflict of interests. Author declares that there is no conflict of interest.

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