J. Plant Production, Mansoura Univ., Vol. 8 (4):473 - 484, 2017

Micropropagation and *In Vitro* Secondary Metabolites Production of *Ocimum* Species. Review Article

Kasem, M. M.
Veget. & Floric. Dept., Fac. Agric., Mansoura Univ.
m_makram2012@yahoo.com

ABSTRACT

Ocimum species have a major importance not just as an aromatic or ornamental plant which utilized for many softscape purposes, additionally as a medicinal plant because of its high substances from volatile oils and numerous secondary metabolites such as rosmarinic acid, flavonoids and anthocyanins. An overview of the recent studies conducted with the use of plant tissue culture technique for micropropagation by using the direct way like cell, tissue and organ culture or the indirect one as, callus redifferentiation and somatic embryogenesis were reviewed in this paper, because of the genetic variability could occur during the sexual propagation method (seeds). In the same time, production of the important secondary metabolites from *Ocimum* species by using different methods such as organs culture, cells suspension, cells elicitation, bioreactors and cell immobilization were also reviewed.

Keywords: Micropropagation, Secondary metabolites production, Ocimum

INTRODUCTION

Ocimum species are standout amongst the most financially critical therapeutic plants on the planet (Saha *et al.*, 2010). It has a place with the family Lamiaceae, sub-family Ocimoideae and incorporates more than 150 unique species and varieties (Hameed *et al.*, 2015) distributed in tropical districts of Africa, Asia, South and Central America (Labra *et al.*, 2004). The real trouble in the utilization of Lamiaceae species for pharmaceutical purposes lies in the hereditary and biochemical heterogenecity (Dode *et al.*, 2003). The ordinary technique for proliferation of this family is through seeds, however the seed viability is exceptionally poor and low seed germination frequency confines it's propagation on a large scale and the seedling progeny likewise indicate cross pollinated nature of the plant (Heywood, 1978). *In vitro* micropropagation is a successful means for fast multiplication of species in which it is important to get a high offspring uniformity. Thusly, the enthusiasm for utilizing these techniques for fast and large scale of aromatic and medicinal plants has been essentially expanded (Sahoo *et al.*, 1997). Numerous *in vitro* studies have been directed on Lamiaceae species, including the *Ocimum* genus, utilizing different explants, as nodal portions (Shahzad and Siddiqui, 2000), young inflorescence (Singh and Sehgal, 1999), axillary buds (Begun *et al.*, 2000) and leaf explants (Phippen and Simon, 2000). A high level of polymorphism in the genus *Ocimum* produces countless number of species, different varieties like O. *basilicum* L., O. *sanctum* L., O. *gratissimum* L., O. *kilimandscharicum* Gurke, O. *americanum* L., O. *citriodorum* Vis., *O. angustifolium* Benth., O. *burchellianum* Benth., O. *campechianum* Mill., and O. *minimum* L. These species are contrasted in creating essential oils which shifting in the main chemical constituents, but the major components of their essential oil were observed to be alcohol monoterpenes as appeared in Table (1). This is critical since monoterpenes are known to act as anti-carcinogenesis in the initiation phase, in addition to the promotion/progression phases (Bayala *et al.*, 2014). The prevalent constituents in *Ocimum* species essential oils were monoterpene derivatives (linalool, camphor, 1,8-cineole, geraniol, limonene and citral), phenyl propanoid derivatives (methyleugenol, eugenol, methylchavicol, chavicol, methylcinnamate) and sesquiterpenoids like bergamotene, bisabolene and caryophyllene (Tchoumbougnang *et al.*, 2006; Verma *et al.*, 2013; Zheljazkov *et al.*, 2008). Limonene is one such monoterpene that prevents liver, lung, mammary and different cancers types. Generally, these *Ocimum* species have been widely used in food and perfumery industries (Telci *et al.*, 2006; Flanigan and Niemeyer, 2014; Hadian *et al.,* 2014). The aerial parts of the plants are considered as stomachic, antispasmodic and carminative in local pharmaceutical (Sajjadi, 2006). In addition, *Ocimum* spp. are certify with differing therapeutic properties, and broadly utilized as a part of traditional and indigenous medications for the treatment of colds, abdominal pains, measles, coughs, rheumatism, insomnia, gonorrhea, sunstroke, snake bite, insect bites , kidney and stomach malfunctions etc. (Makinen and Paakkonen, 1999). Moreover, the fixed seed oil of *Ocimum* spp. was found to have guarantee for nourishment and bio-refinery industries (Kakaraparthi *et al.*, 2015).

Secondary metabolites are known for their important role in the adaptation of the various plants to their environmental condition. In the meantime, it produces vital pharmaceutical components for the human requirements. Diverse methodologies which used the *in vitro* techniques for enhancing production of these secondary metabolites have been widely examined. The compounds which obtained from the intact medicinal plants or the *in vitro* cell, tissue and organ culture might be specifically utilized as drugs, without any changes, or such compounds may undergo extra semi synthetic modulation (Bourgaud *et al.*, 2001). Rosmarinic acid (RA) is the most common basil phenolic compounds. Moreover, the antioxidant activity of RA is more vigorous than that of vitamin E (Lin *et al.*, 2002). Besides that, different phenolic acids were found in addition to RA, such as vanillic, chicoric, benzoic, p-coumaric, syringichydroxybenzoic, ferulic, caffeic, gentisic and protocatechuic acids (Lee and Scagel, 2010; Tarchoune *et al.*, 2012).

So, the aim of this review is to summarize the recent studies which conducted in the different micropropagation techniques of *Ocimum* species, besides production of the important secondary metabolites of this aromatically and medicinal plant species through the *in vitro* elicitation, organ culture, cell suspension, bioreactors and cell immobilization. This review article could be summarizing as follows;

1: Micropropagation of *Ocimum* species.

The micropropagation technique could be performed by using three ways; the first one is through the direct micropropagation, since there is no callus phase in that process. The second one is through using the indirect micropropagation, as the explants were going to perform callus cells before the organogenesis and the regeneration process. The last one is by using the somatic embryogenesis pathway, as the somatic cells are interring into embryonic phase and finally it produces a somatic embryo which is very similar to the zygotic one. All these micropropagation ways of *Ocimum* species were summarized in the following stages;

Table 1. Essential oils chemical composition of different *Ocimum* species from various world locations.

Plant species	Main second metabolites (%)	Authors	Country
O. *basilicum*	Methyl chavicol (>50), Linalool (>50) andGeraniol (>50)	Abduelrahman *et al.* (2009)	Sudan
O. *gratissimum*	Eugenol (71.65)	Pessoa *et al.* (2015)	Brazil
O. *basilicum*	Linalool (48.3), Methyl chavicol (0.6),Methyl eugenol(1.2),Eugenol (10.1) and Cadinene (7.1)	Telci *et al.* (2006)	Turkey
O. *minimum*	Geranyl acetate (69.48), Terpinenol (2.35) and Octanyl-acetate (0.72)	Ozcan and Chalchat (2002)	Turkey
O. *basilicum*	Linalool (56.7: 60.6), Cadinol (8.6: 11.4), Bergamotene (7.4: 9.2) and Cadinene (3.2: 5.4)	Hussain *et al.* (2008)	Pakistan
O. *basilicum*	Linalool, Methyl chavicol, Eugenol	Marotti *et al.* (1996)	Italy
O. *canum*	Linalool (44.9) and Geraniol (38.2)	Ngassoum *et al.* (2004)	Cameroon
O. *canum*	1,8-cineole (60.1) and cis, transpiperitol (68.5)	Bassole *et al.* (2005)	Burkina Faso
O. *basilicum*	Linalool (69.00), Eugenol (10.00), (E)-α-Bergamotene (3.00), Thymol (2.00)	Keita *et al.* (2000)	Guinea
O. *americanum*	Neral (27.9), Geranial (37.7) and Camphor (38.6)	Mondell *et al.* (2002)	Bangladesh
O. *gratissimum*	Thymol (58.2)	Yusuf *et al.* (1998)	Bangladesh
O. *basilicum*	Linalool (10.00), Methyl chavicol (60.30), Methyl cinnamate (6.30)	Kasali *et al.* (2005)	Nigeria
O. *basilicum*	linalool (43.5), methyl chavicol (13.3) and 1,8-cineole (6.8)	Chenni *et al.* (2016)	Egypt
O. *basilicum*	Methyl chavicol (52.40), Linalool (20.10), Epi-α-cadinol (5.90), Trans-α-bergamotene (5.20)	Sajjadi (2006)	Iran
O. *sanctum*	Methyl eugenol (67.80), E-Caryophyllene (17.10)	Awasthi and Dixit (2007)	Northern India
O. *basilicum*	Methyl chavicol (81.82), Ocimene (2.93) and bergamotene (2.45)	Pripdeevech *et al.* (2010)	Thailand
O. *basilicum*	1,8-Cineole (4.00), Linalool (28.60), Estragole (21.70), (E)-Methyl cinnamate (14.30)	Politeo *et al.* (2007)	Austria
O.*campechianum*	Eugenol (32.20: 60.60), Methyl eugenol (60.60: 69.500), 1,8-Cineole (0.90:19.70), Elemicin (0.20: 65.90)	Zoghbi *et al.* (2007)	North Brazil
O. *basilicum*	Estragole (85.50), Linalool (1.71)	Koba *et al.* (2009)	Togo
O. *tenuiflorum*	Eugenol (84.00) and β-Caryophyllene (6.90)	Sastry *et al.* (2012)	Deccan Region, India

Direct and indirect micropropagation.

Establishment and explants sterilization.

Explants gathered from field developed plants are generally polluted by different microorganisms. The method of *in vitro* propagation of plant cells or organs is basically committed to take care of two fundamental problems. Firstly, to keep the plant cells and organs free from microorganisms like fungi and bacteria and furthermore, to enhancing the desired differentiation and development in the cells, tissues or organs by providing the suitable media composition and other physical conditions. The interest in choosing free-diseases explants from the mother plants and following the right sterilization methods could prevent the first problem. So, numerous scientists attempted different sterilization methods for surface sterilization of many explants types from *Ocimum* species as shown in Table (2). In addition, explants (axillary buds, leaves or inflorescences) which obtained from mother plants grown in the greenhouses or the field are more generally utilized as segments to establish sterile cultures. Pre-sterilization washes were done in a portion of the reviews and were variable in concentration and time. For instance, develop shoot and leaf tips of O. *sanctum* were washed with savlon (1%) and sterile distilled water for 20 minutes before the sterilization process (Banu and Bari, 2007) and axillary buds from O. *basilicum*, O. *americanum*, O. *sanctum* and O. *gratissimum* were washed for 5 minute in sodium hypochlorite and laboline solution, then water rinse before disinfecting (Mandal *et al.*, 2000). Also, Pattnaik and Chand (1996) used axillary buds of O. *sanctum*, O. *americanum*, and O. *canum* and only did a prewash with water before applying the surface sterilization with a combination of sodium hypochlorite (NaOCl 10.5% v/v), sodium hydroxide (NaOH 0.5%, w/v), sodium chloride (NaCl 10%, w/v) and sodium carbonate (Na_2CO_3 0.3%, w/v) for 10 minutes and finally washing the explants several times by sterile distilled water. On the other hand, Xiong *et al.* (2009) employed a 30 minute pre-rinse in distilled water, followed by a 25% clorox solution for 10 minutes for sterilizing and culturing the leaves of O. *sanctum* on the initiation medium. Jamal *et al.* (2016) observed practically feasible surface disinfection for explants of O. *sanctum* with using $HgCl_2$ (0.1% w/v) for 7 min. or NaOCl (1% v/v).What's more, they showed that the nodal explants are more suitable to micropropagation than the shoot tips. Besides that, young leaf explants from O. *basilicum* at different development stages (cotyledons, petioles and stems) were service sterilized with 20% bleach (Clorox) for 20 min (Phippen and Simon, 2000). In addition, Mishra (2015) sanitized the recent leaves of O. *sanctum* under running faucet water for 20 min. and were surface disinfected independently in an aqueous solution of

HgCl2 (0.1% w/v) for 3-4 minutes. While, small inflorescences (0.6-1.2 cm) from develop plants of O. *sanctum* were disinfected in 0.2% HgCl2 solution (with 5 drops of Tween-20 per 100ml) for 6-9 min., followed by washing several times with sterile distilled water (Singh and Sehgal, 1999). Moreover, shoot apexes of O. *basilicum* from mature plant (1.0 cm long) were washed and sterilized with ethanol (70% v/v) for1min., then rinsed in sterile distilled water, followed by the main disinfectant solution of NaOCl (0.4%) for twenty min with washing five times in sterile distilled water to overcome the residual effect of sodium hypochlorite (Baroli *et al.,* 2016). Additionally, nodal explants from *O. basilicum* (1-2 cm) were collected from 2-3 month old mother plants and washed under running faucet water, afterward treated with 5% Teepol for 15-20 min., followed by flushing several times with sterile distilled water. Followed by, the surface sterilization with 0.1% HgCl2 solution for 5-6 min (Saha *et al.,*2010). Besides that, Ekmekci and Aasim (2014) used O. *basilicum* seeds as explant and disinfected it by 2.5% NaOCl (commercial bleach) for 10 min., followed by continuous washing with sterile distilled water for 5 min. Moreover, Bhuvaneshwari *et al.* (2016) sterilized nodes, stems, leaves and inflorescences from three months old branches of O. *tenuiflorum* and O. *basilicum* under running tap water for 45 min, then with 1% (v/v) Teepol for 10 min. the main chemical sterilizer was HgCl2 at 0.1%(w/v) for 3 min.

Table 2. *In vitro* sterilization methods of different *Ocimum* species explants.

Ocimum species	Explant type	Main sterilization detergent	Sterilization duration (min)	Authors
O. *americanum* O. *sanctum* O. *canum*	Axillary buds	$HgCl_2$ (0.1% w/v) followed by NaOCl (10.5% v/v), NaCl (10% w/v), NaOH (0.5% w/v) and Na_2CO_3 (0.3% w/v).	5 10	Pattnaik and Chand (1996)
O. *sanctum*	Young inflorescences	$HgCl_2$ (0.2% w/v)	6-9	Singh and Sehgal (1999)
O. *americanum* O. *basilicum* O. *gratissimum* O. *sanctum*	Axillary buds	Laboline (5%) and NaOCl (7% v/v)	5	Mandal *et al.* (2000)
O. *basilicum*	Cotyledons, petioles and stems	Bleach (20% v/v)	20	Phippen and Simon (2000)
O. *basilicum*	Seeds	Commercial Clorox (40%)	20	Kasem (2006)
O. *sanctum*	Mature shoots and leaf tips	Savlon (1% w/w)	20	Banu and Bari (2007)
O. *sanctum*	Leaves	Clorox (25% v/v)	30	Xiong *et al.* (2009)
O. *basilicum*	Nodal explants	$HgCl_2$ (0.1% w/v)	5-6	Saha *et al*. (2010)
O. *basilicum*	Seeds	NaOCl (2.5% v/v)	10	Ekmekci and Aasim (2014)
O. *sanctum*	Young leaves	$HgCl_2$ (0.1% w/v)	3-4	Mishra (2015)
O. *basilicum*	Shoot apexes	NaOCl (0.4% w/v)	20	Baroli *et al.* (2016)
O. *basilicum* O. *tenuiflorum*	Branches	$HgCl_2$ (0.1 w/v)	3	Bhuvaneshwari et al. (2016)
O. *sanctum*	Nodal segments and shoot tips	$HgCl_2$ (0.1% w/v) or NaOCl (1% v/v)	7	Jamal *et al.* (2016)

For shoot initiation, Saha *et al.* (2010) cultured excised nodal fragments (1- 1.2 cm) from O. *basilicum* on Murashige and Skoog (1962) basal medium containing 3% (w/v) sucrose and pH of 5.7 before gelling with 0.8% (w/v) agar. The explants were planted vertically on the growth medium in test tube (150 × 25 mm) and closed firmly with non-absorbent cotton. Also, Mishra (2015) cut the sanitized young leaves of O. *sanctum* into 5mm x 5mm squares and these explants were immunized on MS basal medium for the same objective.

Induction of callus and shoot multiplication

For enhancing cloning techniques, getting various plantlets from an individual explants knows as the multiplication rate. This procedure requires the supplementation of different plant growth regulators to empower shoot multiplication and development of the *in vitro* propagated plants. Data in Table (3) summarize the effect of different plant growth regulators on the callus and shoots multiplication which obtained from various researchers. Shoots multiplication could achieve through the direct or indirect proliferation. For instance, nodal explants with axillary buds could prolong and produce *in vitro* shoots, which can be subcultures for further cloning of plants (George *et al.*, 2008). Likewise, adventitious shoots can be derived specifically from explants or indirectly through callus cultures. Indirect proliferation is generally unfavorable for the aromatic or medicinal plants micropropagation, as the callus development can prompt somaclonal variation or hereditary instability. The *in vitro* shoot cultures are usually kept on media fortified with naturally cytokinins such as, kinetin and zeatin or synthetic cytokinins like BA and TDZ (Jones *et al.*, 2007). Plant growth regulators type and concentration varies depending on the plant species, growth stage, the explants age and size. This is because of the variation in the concentration of the endogenous hormones influencing the prerequisites to energize the multiplication process (George *et al.*, 2008).

In this area, young inflorescences explants of O. *sanctum* which cultured on media fortified with TDZ or 2,4-D formed just non morphogenetic callus. Other than that, using BAP (1.0 mg/l) alone or in combination with IAA (0.05 mg/l) delivered the greatest regenerated shoots (Singh and Sehgal, 1999). Similarly, Hakkim *et al.* (2007) added 2,4-D (1.0 mg/l) in combination with KIN to the development medium of O. *sanctum* and got a high response for callus formation from it. Likewise, nodal explants were gone to produce callus and shoots with the combination of NAA at 5 mg/l and BA at 0.5 mg/l or 2,4-D at 0.2 mg/l (Shahzad and Siddiqui, 2000). What's more, shoot tips from O. *sanctum* plants which cultured on medium invigorated with 0.1 mg/l NAA and 0.2 mg/l BA recorded a higher multiplication rate. However, callus

subcultured onto medium received 1.0 mg/l BA recorded the highest organogenesis callus induction frequency of 90%. While, MS medium fortified with only BA at 0.2 mg/l produced the superior shoot regeneration percentage of 80% (Banu and Bari, 2007). Additionally, axillary buds from O. *tenuiflorum* created the greatest shoots number on medium containing 13.42µM NAA and 2.32µM KIN. Other than that, callus development is very shifted according to the explants types and the growth regulator. Nonetheless, the mother plant and the harvesting time may assume an essential role in deciding the optimal concentration of the plant growth regulator for the indirect callus formation. Besides that, callus from axillary buds required higher auxins levels with low cytokinin levels to improve and increase the shoots and leaves number (Gogoi and Kumaria, 2011).

Table 3. Response of *Ocimum* species to the *in vitro* growth media composition on callus and proliferation formation.

Ocimum species	Explant type	Media composition	*In vitro* response	Authors
O. *sanctum*	Axiliary shoots	BA (1.0 mg/l)	Sm	Pattnaik and Chand (1996)
O. *sanctum*	Young inflorescence	BAP (1.0 mg/l) alone or with IAA (0.05 mg/l)	Sm	Singh and Sehgal (1999)
O. *sanctum*	Young inflorescence	2,4-D (0.5 mg/l) or TDZ (0.05 or 0.5 mg/l)	C	Singh and Sehgal (1999)
O. *basilicum*	Leaves	TDZ (16.8 µM)	C and Sm	Phippen and Simon (2000)
O. *sanctum*	Nodal segments	NAA (5 mg/l) and BA (0.5 mg/l) or 2,4-D (0.2 mg/l)	C and Sm	Shahzad and Siddiqui (2000)
O. *basilicum*	Nodal explants	BAP (0.2 mg/l)	Sm	Begum *et al.* (2002)
O. *basilicum*	Cotyledons	BAP (5.0 mg/l) + NAA (0.2 mg/l)	Sm	Dode *et al.* (2003)
O. *gratissimum*	Nodal explants	BAP (0.5 mg/l) and IAA (0.25 mg/l)	Sm	Gopi *et al*. (2006)
O. *basilicum*	Node with a pair of axillary buds	BAP (1 mg/l)	Sm	Kasem (2006)
O. *sanctum*	Shoot tips	NAA (0.1 mg/l) and BA (0.2 mg/l)	Sm	Banu and Bari (2007)
O. *sanctum*	Leaves, stems and inflorescences	2,4-D (1.0 mg/l) + KIN (0.1 – 0.5 mg/l)	C	Hakkim *et al.* (2007)
O. *basilicum*	Shoot tips	PGR-free MS medium	Sm	Siddique and Anis (2008)
O. *sanctum*	Leaves	NAA (2.0 mg/l) and KIN (0.2 mg/l)	C	Shilpa *et al.* (2010)
O. *tenuiflorum*	Axillary buds	NAA (13.42µM) and KIN (2.32µM)	C and Sm	Gogoi and Kumaria (2011)
O. *basilicum* O. *sanctum* O. *gratissimum*	Cotyledons	2,4-D (1.0 mg/l) and BA (0.5 mg/l)	C	Mathew and Sankar (2011)
O. *sanctum*	Young leaves	Picloram (3mg/l) and BA (1.0mg/l) + IAA (0.5 mg/l)	C and Sm	Mishra (2015)
O. *sanctum*	Shoot tips and nodal segments	BAP (2.0 mg/l) + NAA (0.5 mg/l)	Sm	Jamal *et al.* (2016)

Sm; Shoot multiplication, C; Callus

Cotyledons have also been used as explants source for callus formation on MS nutrient medium fortified with 2,4-D and BA (Mathew and Sankar, 2011). In addition, Shilpa *et al.* (2010) confirmed that the leaf explants are the most common source for callus initiation in holy basil. Moreover, young leaves of O. *sanctum* produced callus cells within 7-10 days by utilizing 3.0 mg/l picloram and the best response to shoot induction with greatest shoot lengthening 6.8cm was gotten by utilizing 1.0 mg/l BA in combination with 0.5 mg/l IAA. Additionally, this medium recorded 82% shoot bud multiplication with 23.8 mean shoots numbers (Mishra, 2015). While, nodal explants of O. *gratissimum* which cultivated on MS medium braced with 0.5 mg/l BAP reacted well contrast with KIN for shoot proliferation and IAA was more viable comparing with IBA. Since, the greatest shoots multiplication induction (14.3) was accomplished from medium fortified with 0.5 mg/l BAP and 0.25 mg/l IAA, with 6.8cm shoot length (Gopi *et al.*, 2006). Siddique and Anis (2008) gained an increase in shoot elongation and multiplication values after the third subculture of O. *basilicum* on MS free hormones medium. Also, Phippen and Simon (2000) cleared that the explants age is very important factors influencing the regeneration frequency, since the highest multiplication percentage of 85% with 5.1 shoots /explants was obtained when one month old seedlings from O. *basilicum* were used as explants. In addition, a negative relationship between the explants age and the organogenesis response was found in their study. On the other hand, fortifying the growth media with BA at 1.0mg/l increased the shoot proliferation percentage when axillary shoot buds of O. *sanctum* were cultured on it (Pattnaik and Chand, 1996). Moreover, Jamal *et al.* (2016) obtained the greatest shoots number of O. *sanctum* by culturing the explants on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA.

***In vitro* rooting and acclimatization of plantlets**

Roots formation is very difficult process in many plants (Custodio *et al.*, 2004) and is adapted by various factors such as biochemical, physiological and genetic (Pawlicki and Welander, 1995). In addition, Thorpe (1982) adumbrated that initiation and growth of adventitious roots were high energy acute processes that need some metabolic substrates, fundamentally the carbohydrates beside the growth hormones. Siddique and Anis, (2008) observed the greatest rooting frequency of O. *basilicum* on MS basal medium which fortified with 1.0µM IBA. Also, Sahoo *et al.* (1997) confirmed that roots formation of the excised shoots from O. *basilicum* were not achieved on MS half strength free hormones medium even after 30 days from the *in vitro* culturing. Besides that, they added that IBA was more suitable and effective comparing with the other tested auxins. Similary, Daniel *et al.* (2010) reported that 1.5 mg/l IBA alone in the rooting media of O. *basilicum*, consider the effective auxin in producing the highest rooting percentage (89%) and the rooted plantlets were successfully acclimatized and transferred to the natural condition with high survival percentage (90%). Dode *et al.* (2003) found that addition of NAA in the *in vitro* rooting medium of O. *basilicum* prevented the

formation of roots when combined with different auxins concentrations. While, Asghari *et al.* (2012) observed a negative relationship between increasing the concentration of BA in the rooting media and the roots formation from the nodal or the cotyledons explants of O. *basilicum*, whereas the matter was differ with the hypocotyl explants, since every increase in BA, followed by increase in the roots formation. Moreover, they added that IAA at 2.85µM was more effective in producing the highest roots formation percentage. While, Jamal *et al.* (2016) stated that O. *sanctum* regenerated shoots were produced a vigorous roots in full MS media fortified with 1.0 mg/l NAA. On contrast, Sehgal and Singh, (1999) expressed that 90% of the *in vitro* regenerated shoots formed roots on MS free hormones medium through 2-3 weeks of the culture process. Other than that, the survival rates of the *in vitro* developed plantlets of basil are high with a short adaptation procedure. Moreover, transplanting longer rooted shoots into mixture of garden soil and vermiculite (1:1v/v), produced a high survival percentage (85%). Likewise, *in vitro* shoots of O. *sanctum* were rooted on MS medium supplemented with 1.5mg/l IBA and the rooted plantlets were effectively acclimatized and set up under common conditions with 90% survival percentage (Mishra, 2015).

George *et al.* (2008) illustrated that the *in vitro* raised plantlets frequently require acclimatization before the transplanting to the nursery (greenhouses) or the field conditions as they adapted to high humidity in the *in vitro* condition, with little cuticle layer on the leaves or the total herb and poorly differentiated stomata. In addition, the plantlets may not be totally autotrophic because of the sugar supply in the growth media. In this way, the *in vitro* developed plantlets are removed from the *in vitro* culture conditions, washed and transferred to the greenhouse with high dampness through moistening and low light intensity. After a moderate acclimatization through decreasing humidity and expanding the light intensity, the plantlets turn out to be completely autotrophic and set up to survive independently. Sharma and Kumar (2012) added 1.0 mg/l IBA to the half strength MS rooting medium of O. *minimum*, and transferred the well rooted plantlets on a mixture of sand, garden soil and farm yard manure (1:1:1 v:v) and they recorded the highest survival percentage (70-80%) on that mixture. In another acclimatization method, Pattnaik and Chand (1996) moved O. *americanum*, O. *canum* and O. *sanctum* plantlets into development chambers with high dampness for 2 weeks before exchange to the greenhouse with survival percentage of 75-80% in the development chamber, 80-85% after exchange to the greenhouse followed by 100% survival in the open field conditions. On the other side, Gogoi and Kumaria (2011) found a variable survival percentage in various soil compost mixtures for O. *sanctum* ranged from 12 to 82% during the acclimatization process. As, the greatest survival rate was obtained from using mixture of soil and cow dung and the lowest one was recorded for using the compost acclimatization media.

Somatic embryogenesis

Somatic embryogenesis is the procedure by which somatic cell develop into somatic embryo after a concatenation of some morphological and biochemical changes. These embryos are morphologically like the zygotic embryos (Quiroz-Figueroa *et al.*, 2006). The procedure of somatic embryos includes a series of stages: embryogenic callus creation, formation of somatic embryos, maturation of somatic embryos and transmutation into plantlet. However, the accomplishment of this procedure relies on upon a several elements, including explants type, genotype, and plant growth regulators. Among the plant growth regulators (PGRs), auxins are known to be basic for somatic embryogenesis induction.

In many plant species 2,4-D is the most ordinarily utilized auxins. Several reviews in production of *Ocimum* somatic embryos were achieved. Mathew and Sankar (2011) cultured the cotyledonary leaves of O. *basilicum*, O. *gratissimum* and O. *sanctum* for generation of somatic embryos and found that MS medium fortified with 2,4-D (1.0mg/l)+ BA (0.5 mg/l) was appropriate for the embryonic callus development with greatest weight and few days for induction of O. *sanctum* and O. *basilicum*. Whereas, MS medium supplemented with 2,4-D (0.5 mg/l) + BA (0.5 mg/l) was suitable for O. *gratissimum*. The differentiation of somatic embryos into the globular phase was observed in the entire cultured media which contained different combinations and concentrations from KIN, BA and IAA, but with variation in the induction duration and the embryos color. Also, the greatest differentiation percentage of somatic embryos into the globular shape was recorded by culturing on medium fortified with KIN with or without IAA and BA at 2.0 or 3.0 mg/l. While, Hakkim *et al.* (2011 a) cultured inflorescences, leaves and stems explants of O. *sanctum* for induction of embryonic callus and they found that combination of NAA (1.0mg/l), BAP (1.0mg/l) and KIN (0.5mg/l) produced the greatest embryogenesis potential of 73% and globular embryos number of 34.3/callus. Besides that, Gopi and Ponmurugan (2006) developed powerful protocol for O. *basilicum* through somatic embryogenesis. Since, leaf derived callus was initiated on MS medium supplemented with 1.0 mg/l 2,4-D and this callus differentiated into globular embryo structure when transferred to medium contained 0.5mg/l 2,4-D and 1.0mg/l BAP. In addition, the highest differentiation percentage of the globular embryos was observed on medium having 1.0mg/l BAP + 1.0mg/l NAA + 0.5mg/l KIN with 80% survival percentage when the germinated embryos transferred to the *ex vitro* condition. What's more, Livadariu (2011) recorded the greatest embryogenesis response on MS medium contained1.0mg/l TDZ and 0.5 mg/l IBA when the cotyledon was the cultured explants.

2: Secondary metabolites production via plant cell and tissue culture

Plants create large quantities of compounds that are not entirely vital for development and growth, but rather which assume an essential role in resistance and adaptation to the environmental conditions. These gatherings of organic compounds that are created by the plants to encourage the interaction with the biotic conditions are called plant secondary metabolites (Murthy *et al.*, 2014). The production of these secondary metabolites is low (under 1% DW) and depends significantly on the

development stage and the physiological phase of the plant (Oksman-Caldentey and Inze, 2004). Moreover, these secondary metabolites include phenolics, terpenes, alkaloids and steroids which have massive imaginable purposes as agrochemicals, pharmaceuticals, fragrances, flavors, pesticides, food additives and colors (Cusido *et al.*, 2013 ; Gupta *et al.*, 2010). *Ocimum* species are much esteemed for their powerful antifungal, antibacterial, therapeutic and antioxidant activities. These imperative secondary metabolites could be enhanced and produced via plant tissue culture through many ways (as shown in Table 4) such as the organ and callus culture, cells suspensions, precursors and elicitors addition to the growth media, bioreactors and immobilization of the plant cells.

Table 4. Summarization of the different *in vitro* plant tissue culture techniques for production of *Ocimum* species secondary metabolites.

Ocimum species	Secondary metabolite	Media composition	Method of production	Authors
O. *basilicum*	Total oil yield and linalool	2,4-D (0.1 mg/l)	Cell suspension	Purohit, and Khanna (1983)
O. *basilicum*	Rosmarinic acid and lithospermic acid	MS free hormone medium	Hairy root	Tada *et al.* (1996)
O. *basilicum*	Rosmarinic acid	fungal cell wall elicitor (*Phytophthora cinnamon*)	Hairy root	Bais *et al.* (2002)
O. *basilicum*	Rosmarinic acid	Phenylalanine (0.5 g/l), 2,4-D (2 mg/l) and NAA (2 mg/l)	Cell suspension	Kintzios *et al.* (2003)
O. *basilicum*	Rosmarinic acid	-	Nodal explants in bioreactor	Kintzios *et al.* (2004)
O. *basilicum*	Rosmarinic acid and eugenol	Chitosan		Kim *et al.* (2005)
O. *americanum*	Rosmarinic acid	BA (1.0 mg/l) and IAA (0.25 mg/l)	*In vitro* regenerated plantlets	Rady and Nazif (2005)
O. *basilicum*	Cineol, linalool, terpeniol and Methyl Chavicol	BA (5 mg/l)	*In vitro* regenerated plantlets	Kasem (2006)
O. *basilicum*	Antioxidant	Yeast extract (5 g/l)	Callus	Guirgis *et al.* (2007)
O. *basilicum*	Antioxidant	Irradiation	Callus	Homhuan *et al.* (2008)
O. *basilicum*	Betulinic, oleanolic, ursolic, 3-epimaslinic, alphitolic and euscaphic acids	*Agrobacterium rhizogenes*	Hairy root	Marzouk (2009)
O. *sanctum*	Rosmarinic acid	2,4-D (1 mg/l) and KIN (0.1 mg/l)	Callus	Hakkim *et al.* (2011 a)
O. *sanctum*	Rosmarinic acid	Sucrose (5.0%), phenylalanine (0.25 g/l), yeast extract (0.5 g/l) and methyl jasmonate (100M)	Cell culture	Hakkim *et al.* (2011 b)
O. *basilicum*	Caffeic acid (Rosmarinic acid)	BA	Organ culture (leaves, roots and plantlet shoots)	Kiferle *et al.* (2011)
O. *basilicum*	Rosmarinic acid	Gel matrix	Immobilized cells	Moschopoulou and Kintzios (2011)
O. *basilicum*	Anthocyanins and Rosmarinic acid	-	Cell suspension	Strazzer *et al.* (2011)
O. *basilicum*	Rosmarinic acid	BA (5mg/l) and NAA (1mg/l)	Callus and shoot cultures	Abdel Rahman *et al.* (2015)
O. *basilicum* O. *kilimandscharicum* O. *sanctum* O. *grattisimum*	Betulinic acid	2,4-D, NAA and methyl jasmonate	Callus	Pandey *et al.* (2015)
O. *basilicum*	β-carotene, ascorbic acid, phenolics, flavonoids and gallic acid	2,4-D (0.5 mg/l)	Callus	Wongsen *et al.* (2015)
O. *sanctum*	Eugenol	Phenylalanine (5mg/l)	*In vitro* regenerated shoots	Sharma *et al.* (2016)

Organ and callus Cultures

Organs of the *in vitro* regenerated plantlets could be a good source of many secondary metabolites. Also, the unorganized proliferative mass of cells which produced from isolated plant cells, tissues or organs when grown aseptically on artificial nutrient medium is known as callus cells. Also, this callus when treated with plant growth regulators at different concentrations or elicitors could produce high amounts of secondary metabolites. Along these lines, Hakkim *et al.* (2011 a) recorded the highest biomass growth (17.8 g/l) and rosmarinic acid content (104 mg/l) in growth media supplemented with 2,4-D (1 mg/l) and KIN (0.1 mg/l). It was twofold higher than that found in the leaf callus which stimulated on MS solid medium. While, Marzouk (2009) identified six triterpene acids as oleanolic, betulinic, ursolic, euscaphic, 3-epimaslinic and alphitolic acids which separated from a dichloromethane extract of O. *basilicum* hairy roots cultures. These *in vitro* cultures were gotten by genetic transformation by utilizing *Agrobacterium rhizogenes*. In addition, Rady and Nazif (2005) estimated the rosmarinic acid content of the *in vitro* plantlets of O. *americanum* and they observed that MS medium fortified with 1.0mg/l BA and 0.25 mg/l IAA generated the maximum RA accumulation. Moreover, Abdel Rahman *et al.* (2015) observed that shoot and callus cultures of O. *basilicum* which cultured on medium supplemented with 5mg/l BA and 1mg/l NAA accumulated higher levels of RA (ranged from 9.42 to 38.25g/mg DW) compared to the control. Other than that, Tada *et al.* (1996) outlined that in O. *basilicum* hairy roots cultures, RA was the major phenolic compound. Additionally, Wongsen *et al.* (2015) cultured leaves of sweet basil (O. *basilicum*) on a semi-solid nutrient medium fortified with various 2,4-D concentrations and observed that all the examined media could enhance production of the green compact callus, except for the control treatment

(free PGRs medium). The superior 2,4-D concentration in production the greatest callus fresh weight was 0.5mg/l. Besides that, the antioxidant content analysis of the formed callus had higher levels of ascorbic acid, β-carotene, flavonoids and phenolics (0.64 mg/g F.W., 0.08 mg/g F.W., 7.38 mg rutin/g F.W. and 6.54 mg gallic acid/g F.W., respectively) after one week of the callus proliferation.

By using the gamma rays irradiation, Guirgis *et al.* (2007) and Homhuan *et al*. (2008) confirmed that treating sweet basil callus by applying this irradiation could produce increasing in the antioxidant content. Also, Kiferle *et al.* (2011) recorded caffeic acid derivatives, in particular rosmarinic acid (RA) in different tissues (plantlet shoots, roots and leaves) of O. *basilicum* plants grown either *in vitro* or in floating system of the hydroponic culture under greenhouses conditions. They found that the content of RA ranged from 4 to 63 mg/g D.W., depending on the growing system. The highest RA content was found during the *in vitro* multiplication, in the acclimatized plants and in the roots of hydroponically-grown seedlings at full bloom. *In vitro*, BA (6-benzyladenine) reduced the accumulation of RA in purple-leaf Dark Opal cultivar of sweet basil, but an opposite effect of this growth regulator was observed in the green-leaf genotypes.

Cell suspensions

Suspension culture is a kind of culture in which single cells or small multiplies cells aggregates suspended in rapturous liquid media. This suspension is a standout amongst the most imperative ways for generation and improvement of secondary metabolites. Strazzer *et al.* (2011) chosen a red basil cell line from O. *basilicum* for improving accumulation of rosmarinic acid (RA) and anthocyanins (ACs) in the cell suspension cultures and they identified different rosmarinic acid related molecules, some caffeic acid, coumaric acid and some flavones. Kintzios *et al.* (2003) concluded that the suspension cultures of O. *basilicum* which obtained from leaves accumulated RA over to 10 mg/g D.W., with a value up to 11 times higher than in the donor plant leaves or in the callus cultures. While, Purohit, and Khanna (1983) explored volatile oil production of suspension and callus cultures of O. *basilicum* under continuous illumination and those developed in a complete darkness. It has been demonstrated that the volatile oil yield and the linalool percentage were expanded in the light cultures conditions.

***In vitro* plant cell elicitation**

In order to upgrade secondary metabolites synthesis, many organic compounds could be added to the culture media (Namdeo *et al.*, 2007). The idea depends on the possibility that any compound, which is an intermediate in or at the beginning of a secondary metabolite biosynthetic way, attitude a perfect opportunity of improving and increasing the final product yield (Rao and Ravishankar, 2002). It is trusted that secondary plant products have a primary role in the defense system of the plant. Along these lines, biotic stress as a result of infection by bacteria, virus, fungi, insects and nematodes improves secondary metabolites synthesis. Compounds that trigger the plant cells for production of such secondary metabolites are called as elicitors. Contingent upon their source, they are distributed to biotic or abiotic elicitors (Smetanska, 2008). Biotic elicitors intended from compounds acquired from microbial cell wall, bacterial extracts and fungal extracts have been utilized for improving the secondary metabolites production. Similarly, abiotic elicitors like cells or tissues exposure to low or high temperatures, ultraviolet light radiations, antibiotics, fungicides and heavy metals salts have extremely affected the secondary metabolites accumulation. In spite of the fact that both biotic and abiotic elicitors have been appeared to improve the secondary metabolites, no elicitor has been found to generally affect numerous or all cultivated hairy roots/cells (Jawahar *et al.*, 2014). In this way, Kim *et al.* (2005) treated sweet basil with different chitosan concentrations which improved rosmarinic acid (RA) and eugenol concentrations to 2.5 and 2 folds, respectively. Furthermore, they recommended that elicitor such as chitosan can adequately actuate phytochemicals in plants, which may be another alternative rather than hereditary modification.

Hakkim *et al.* (2011 b) found that cellular content from rosmarinic acid expanded gradually and achieved the most extreme after 18 days of vaccination of O. *sanctum* cell culture with different elicitors. Increasing the sucrose concentration up to 5%, significantly improved accumulation of rosmarinic acid (2.7 fold) comparing with the control treatment which fortified with 3% sucrose. Moreover, supplementing the growth media with 0.25g/l phenylalanine produced higher rosmarinic acid accumulation (4.1 fold) comparing with the non-supplemented media, since rosmarinic acid content was 5.7 mg/l/day. In the same time, adding 0.5g/l yeast extract increased rosmarinic acid content up to 7.0 fold comparing with the non-elicited suspension cells. Furthermore, a dramatic increment in cell growth and RA accumulation (8.6 fold) were observed with elicitation by 100M methyl jasmonate comparing with the non-elicited cells. Also, Gleba *et al.* (1999) confirmed that elicitors stimulate many plants roots to exude phytochemicals in extremely higher amounts than non-elicited plants. While, Pandey *et al.* (2015) looked at the capacity of four *Ocimum* species (O. *kilimandscharicum*, O. *basilicum*, O. *grattisimum* and O. *sanctum*) for induction of betulinic acid in the callus cells, comparing with the *in vitro* derived leaves. Callus formations were gained in all the four *Ocimum* species with various NAA or 2,4-D concentrations combined with KIN. Prominently, 2,4-D favored most extreme callus growth, while NAA demonstrated advantageous for producing the highest betulinic acid yield in the callus cells. Likewise, the O. *basilicum* callus gave the greatest growth index (GI 678.7) and betulinic acid yield (2.59% DW) though those in O. *kilimandscharicum* (GI 533.3; betulinic acid 1.87% DW) and O. *sanctum* (GI 448; betulinic acid 0.39% DW) followed a descending order. The callus of O. *gratissimum* produced the least growth index (GI 159) with no betulinic acid formation. In addition, elicitation with 200µM methyl jasmonate after 48h duplicated the betulinic acid yield (5.10% DW) in growth medium contained NAA of O. *basilicum* callus comparing with the untreated one (2.61% DW).

Bais *et al.* (2002) found that elicitation of O. *basilicum* hairy root cultures with fungal cell wall elicitors which derived from *Phytophthora cinnamon*, improved rosmarinic acid accumulation (2.67fold) comparing with the control. Meanwhile, use of jasmonic acid, salicylic acid and chitosan had negative effects on the growth of the hairy roots cultures with resulting darkening of the tissues in conjunction with the decreased titers of intracellular RA. On opposite, this result infers that JA, SA and chitosan don't channel RA elicitation. Likewise, Guirgis *et al.* (2007) observed a quickest callus growth of O. *basilicum* on medium fortified with 1 mg/l 2,4-D and 0.25 mg/l KIN. In addition, adding 5 g/l yeast extract to the growth medium enhanced the gathering of RA (3.4 fold). While, Sharma *et al.* (2016) recorded a higher biomass growth and eugenol content on growth medium supplemented with 5mg/l phenylalanine, since 5 g of field grown shoots comprised 8.5 mg of eugenol and when *in vitro* regenerated shoots were grown on medium contained phenylalanine (5 mg/l), they gained 10.08 mg of eugenol.

Bioreactors and Cell Immobilization

Bioreactor is a vessel in which a chemical procedure which includes organisms or biochemical active constituents derived from such organisms was achieved. These bioreactors are regularly barrel shaped, going in size from liters to cubic meters, and are frequently made of stainless steel. The progressed bioreactor protocol is a key stride towards commercial production of active constituents by the plant cells and tissues cultures. Generally, the basal mission of a bioreactor is to produce ideal conditions for cell metabolism and physiology by managing the different environmental factors. The principle criteria for planning plant cells/organs culture bioreactors ought to consider sufficient oxygen exchange, low shear stress, and great mixture (Zhou *et al.*, 2010 and Zhong, 2011).

In that area, Kintzios *et al.* (2004) cultured nodal explants and leaf derived suspension culture of O. *basilicum* in 5/l airlift bioreactors for 3weeks and gained an increase in suspension fresh weight (2.5 fold), with increasing in RA accumulation (29µg/g DW) in this suspension. Immobilization technique has enhanced the plant secondary metabolites accumulation in the cultured cells. Constantly the growth media are liquid for aperients best growth and absorption of nutrients. In suspension cultures, cells grow quicker and subsequently the accumulation of the secondary metabolites is tardily. Further, cell aggregates have the preferred capacity to gather metabolites over the free floating cells. Thusly, it is indispensable to anchor the plant cells onto some solid support in a lattice such as agar, gel, polyurethane, alginate or poly acrylamide. This procedure of anchoring the plant cells is known as immobilization (Jawahar *et al.*, 2014). Thus, Moschopoulou and Kintzios (2011) found amazing rosmarinic acid (RA) accumulation (20 mg/g dry weight) by immobilizing sweet basil (O. *basilicum*) cells in a high density gel matrix, as it was 1400 times higher than in sweet basil cells immobilized in spherical beads. Moreover, doubling the immobilization matrix volume is followed by increasing in the accumulation of rosmarinic acid by 100 fold. In addition, rosmarinic acid was excreted into the culture medium, since it was harvested without terminating the immobilized cells culture. On contrast, Kintzios *et al.* (2003) represented that the immobilized cells of O. *basilicum* amassed under 5µg/l rosmarinic acid.

CONCLUSION

The sexual propagation through seeds is available in most of *Ocimum* species, but the seedling progeny show variability due to cross-pollinated nature of the plant. This gives the plant tissue culture a promising role for using the micropropagation technique with this important genus. Since, the *Ocimum* genus not only uses as ornamental plant, but also as an important aromatic and medicinal plant, as it contains various important secondary metabolites like rosmarinic acid, betulinic acid and another important constituents. In addition, the *in vitro* culture is using for enhancing and stimulating production of such secondary metabolites overcoming some limitation for the *in vivo* production of these constituents as the climatic condition, the soil problems, pests, diseases and correlation with the planting season. Through the various *in vitro* studies, the explants sterilization process was done by utilizing different chemical detergents like HgCl2, NaOCl or the commercial bleach (Clorox) with different concentration and periods depending on the degree of the surface contamination, explants type and the time of explants collection. For increasing the shoots biomass (multiplication) and rooting induction, different cytokinins and auxins were used solely or in combinations. Moreover, *in vitro* production of the main constituents for that genus was performed by using the organ culture especially the roots, callus culture, cell suspension, cells elicitation, bioreactors and cell immobilization.

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الإكثار الدقيق وانتاج المركبات الثانوية معمليا لأنواع الريحان. مقال مرجعى

محمود مكرم قاسم
قسم الخضر والزينة – كلية الزراعة – جامعة المنصورة- مصر

تمتلك انواع الريحان اهمية كبيرة ليس فقط كونها نباتات عطرية او نباتات زينة تستخدم باعمال التنسيق المتعددة، ولكن ايضا كأحد اهم النباتات الطبية نتيجة لإحتوائها على العديد من المركبات الثانوية بالزيوت الطيارة لأنواعه المختلفة مثل حامض الروزمارينيك وبعض الفلافونيدات والانثوسيانينات. تلخص تلك الورقة المرجعية العديد من الابحاث التى اجريت على اهم انواع الريحان بمجال الاكثار الدقيق سواء بالطريقة المباشرة او الغير مباشرة وذلك لما يسببه الاكثار بالطريقة الجنسية (البذور) من انعزالات وراثية وبالتالى عدم الثبات الوراثى والمحتوى الكيميائى الموجود بها (المركبات الثانوية). وفى نفس الوقت يستعرض اهم الطرق المستخدمة بتكنيك زراعة الانسجة بغرض حث وتشجيع تراكم وانتاج المركبات الثانوية الهامة بانواع الريحان مثل زراعة الاعضاء النباتية و معلقات الخلايا وتحفيز الخلايا والمفاعلات الحيوية واخيرا انماء الخلايا بصورة غير حرة.