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Research Article
Implement of Biotic and Abiotic Stress for Enhancement and Production of Capsaicin in Suspension Cultures of *Capsicum annum* spp.

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Abstract

Background and Objective: Pepper (*Capsicum annuum* L.) is one of the major vegetable and spice crops grown worldwide. This study highlighted the economic and importance process for *in vitro* capsaicin production from pepper plant. As well as to study the effect of MS medium supplemented with 2,4-D in combinations with 0.2 mg L^{-1} Kin. for callus and suspension production was implemented. As well as, the influence of *A. niger* as biotic and methyl jasmonate as abiotic elicitors on capsaicin accumulation was investigated. **Materials and Methods:** For this purpose, callus cultures were prepared from seeds of pepper and subcultured. After 28 days, calli formation (%), fresh weight (g/Jar) and dry weight (g/Jar) was calculated. Cell number and packed cell volume were calculated from their cell suspension. In the end, capsaicin was extracted and colorimetrically quantified. **Results:** The highest percentage of calli formation was recorded with hypocotyl, leaf and root explants, respectively. The MS medium fortified with 3.0 mg L^{-1} 2,4-D+0.2 mg L^{-1} Kin. In addition, the maximum P.C.V was recorded after 16 days of cultivation on the same medium. Furthermore, it was found that fortified MS medium with 1.5% of *A. niger* in combination with 100 µM of methyl jasmonate achieved of cell growth parameters and capsaicin accumulation in significant rate during 16 days of cultivation. **Conclusion:** Fortified of MS medium with 3.0 mg L^{-1} 2,4-D+0.2 mg L^{-1} Kin. showed the optimized medium for both callus and suspension production. Moreover, augmentation of MS medium with 1.5% of *A. niger* in combination with 100 µM of methyl jasmonate enhanced of capsaicin accumulation in significant rate during 16 days of cultivation.

Key words: *Capsicum annuum*, capsaicin, growth dynamics, biotic and abiotic elicitor, *Aspergillus niger*, methyl jasmonate

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

It is well know that Pepper (*Capsicum annuum* spp.) is one of an important horticultural crops and growing worldwide, particularly in Asia[1]. Capsicum consists of approximately 25 wild and 5 domesticated species[2]. The five domesticated species are *C. annuum*, *C. baccatum*, *C. chinense*, *C. frutescens* and *C. pubescens*.

Compounds such as phytochemicals are found in plant foods and have bioactive properties such as improve health. Further, the demand for plants with higher contents of phytochemical compounds is increasing[3-6]. Compounds like carotenoids, ascorbic acid, phenolic, vitamins A and E as well as capsaicinoids are found in the fruits of *Capsicum* spp. These compounds have activities as antioxidant, anticancer and anti-inflammatory[7-8]. The principal compounds is capsaicin derived capsaicinoids. Capsaicin is causing pungency in peppers. Moreover, it is employed as food additives for human and animal nutrition as well as in pharmaceutical applications[2,9-12].

Different workers had investigated the biosynthetic capacity of *in vitro* cultured cells to produce capsaicinoids using, nutrient limitation, immobilized cell cultures osmotic stress, precursors and elicitors[13-16]. It was recommended that the yield of capsaicin has been enhanced by addition of both phenylalanine and isocaproic acid (8-methylnonanoic acid) in immobilized and freely suspended cells of *C. frutescens*, respectively[17]. Supplementation of Phenylalanine and phenylpropanoids in suspension cell cultures of *C. annum* enhanced the accumulation rate of capsaicin[18].

The physiological and morphological responses can be induced in plants and other living organisms using elicitors[12,19,20]. Salicylic acid, oligosaccharides, jasmonic acid and hydrogen peroxide are some of elicitors which used in plants for inducing secondary metabolites production and crop protection[19-21].

After plant infection, the plant defenses resistance mechanisms have achieved[22]. This achievement and activation are accompanied process by change in biochemical activities and associated with the expression of defense against pathogens. The biochemical response which associated with defense is accumulation of phytoalexins[23,24]. Their production is correlated and associated either by pathogen infection or by biotic and abiotic elicitors such as synthetic chemicals, substances from microorganisms, polysaccharides, proteins and cellulose. However, phytoalexins are produced by plants not only in response to interactions with micro-organisms but also with many chemicals, irradiation by ultraviolet light and exposure to the products of microbial metabolites[25].

This investigation is highlighted the economic importance of pepper (*Capsicum annuum*) as well as study the effect of different concentrations from 2,4-D in combination with 0.2 mg L^{-1} Kin. and various concentrations from *Aspergillus niger* as biotic stress and methyl jasmonate as abiotic stress on cell growth and total capsaicin production from different types cultures of *Capsicum annuum* L.

MATERIALS AND METHODS

This investigation was carried out during the period of 2017-2018 at Plant Biotechnology Department, National Research Centre, Dokki, Cairo, Egypt.

Callus cultures: Seeds of pepper (*Capsicum* spp.) have been washed with abundant tap water to eliminate dust and then surface sterilized by immersion in 70% ethanol for 5 min followed by 20 min in 20% (v/v) Colorox (5.25 Cl) containing 0.1% Tween-20. Further, Sterilized seeds were washed with sterilized and autoclaved water. Then they were cultured on half salts strength of MS basal medium[26] and incubate at 25±1°C under photoperiod (16 h light/8 h dark; white daylight fluorescent tubes (50 μmoL m^{-2} sec^{-1}) for 4 weeks. Hypocotyl, leaf and root explants were excised (1 cm in length) from the seedlings and transferred onto glass Jars (100 mL) containing 20 mL of MS basal medium supplemented with different concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D) at the ratio of 0.0, 1.0, 3.0 and 5.0 mg L^{-1} in combinations with 0.2 mg L^{-1} Kinetin (Kin). Three explants were cultured/Jar. Cultures media contains sucrose (3%) as the carbon source. The pH media was adjusted at 5.8 before autoclaving (121°C; 20 min) and the medium was gelled with 0.7% agar. Further, cultures were incubated for 4 weeks under the same conditions described previously for seeds germination and seedlings production. At this time, friable calli tissue were observed at the cut ends of the ex-plant. Obtained calli were subcultured every 28 days by transferring few portions of friable calli tissue onto fresh medium. The following parameters were recorded after 28 days as follow:

- Percentage of calli formation
- Fresh weight (g/Jar)
- Dry weight (g/Jar)

Cell suspensions: Pepper cell suspension cultures were established from friable obtained calli according to the described method[27]. A passage of calli (0.5 g fresh weight)

were recultured into 125 mL Erlenmeyer flasks containing 25 mL of MS liquid culture medium supplemented with 3.0 mg L^{-1} 2,4-D+0.2 mg L^{-1} Kin. Then, cultures were incubate on a rotary shaker (125 rpm) at 25±1°C under photoperiod (16 h light/8 h dark; cool white fluorescent tubes; 50 µE m^{-2} sec^{-1}). Cell suspension cultures were subcultured at 15 days intervals. Maintain the cell cultures were carried out in MS liquid culture medium supplemented with same concentrations of previous growth regulators, pH 5.7.

The following parameters were recorded after 4, 8, 12, 16, 20 and 24 days of cultivation as follow:

- **Cell number:** According to Neumann[28], the cell number was counted during the growth period of cultivation as a growth parameter
- **Packed cell volume:** Packed Cell Volume (PCV) was determined according to King[29] after 4, 8, 12, 16, 20 and 24 days of cultivation

Treatments with elicitor of *Aspergillus niger* as biotic stress

Elicitor preparation: *Aspergillus niger* was obtained from Department of Plant Pathology of the National Research Centre. It was grown in malt extract (20 g L^{-1}) in a shake flask (1000 mL) with 200 mL medium on a rotary shaker (120 rpm) at room temperature as the described method[30]. After 7 days the cell suspension was autoclaved and filtered on Whatman No. 1 filter paper. The mycelium was washed several times with sterilized distilled water and suspended in 100 mL water. This mixture was homogenized, autoclaved again and measured through the PCV and used without purification. In this experiment, different concentrations 0, 0.5, 1, 1.5 and 2.0% of suspended *A. niger* were added to the culture media for 16 days.

Treatments with elicitor of methyl jasmonate as abiotic stress: In this experiment Methyl Jasmonate (MJ) at different concentrations of 0, 50, 100, 150 µM were added to MS medium supplemented with 3.0 mg L^{-1} 2,4-D+0.2 mg L^{-1} Kin+ 15% of 0.1% of PCV of *A. niger* for 16 days of cultivation.

Extraction, separation and quantification of capsaicin: Collected pepper cells were lyophilized and homogenized for 3 min and then a five fold volume of acetone was added, respectively, to the extract at 50°C for 1 h in triplicate. Centrifuged supernatant was taken for colorimetric analysis as method reported[31].

Colorimetric quantification of capsaicin: One milliliter acetone extract of pepper cell suspension cultures were transferred into a glass test tube and completely dried by liquid nitrogen. Five milliliters n-hexane was added and the mixture was allowed to remain at room temperature for 10 min to dissolve the extract. Then, the 4 mL n-hexane layer was carefully taken to a new tube without any solid materials tagging along. About 4 mL of n-hexane solution,10 mL of 0.05N NaOH was added and the mixture was intensely vortexed. The supernatant was removed. One milliliter from the NaOH layer left was taken and then mixed with 50 mL of 1N HCl, 50 mL of 0.1% 2,6-Dichloroquinone-4-chloromide (DCQ) and 50 mL of 2.5% ammonia solution in order. The mixture was reacted at room temperature for 10 min and the absorbance was measured at 600 nm.

For the standard, 5 mg capsaicin was dissolved in 10 mL of methanol and 50, 100, 200 and 400 mL of the solution were transferred into glass tubes with the methanol removed by nitrogen gas. Each standard was dissolved in 4 mL of hexane and then extracted with the NaOH solution for color development as mentioned earlier.

Statistical analysis: Design of all experiments was completely randomized and the obtained data were statistically analyzed using Standard Error (SE) according to the method reported [32].

RESULTS

Callus induction: In this experiment segments of hypocotyl, leaf and root explants were cultured on MS medium supplemented with different concentrations of 2,4-D 0.0, 1.0, 3.0 and 5.0 mg L^{-1} in combinations with 0.2 mg L^{-1} Kin. all cultures were incubated under light conditions. Data in Table 1 clearly showed that the highest percentages of callus formation 95.6, 86.3 and 71.6 were recorded with hypocotyl, leaf and root explants, respectively. Moreover, the maximum fresh and dry weights 2.25, 1.89, 1.35 and 0.17, 0.14, 0.08 g/jar were recorded with hypocotyl, leaf and root explants, respectively. The best supplementations to MS medium for callus production were 3.0 mg L^{-1} 2,4-D+0.2 mg L^{-1} Kin compared other supplementations. Furthermore, the hypocotyl ex-plants (Fig. 1) more sustainable for maximum calli production compared leaf or root explants, respectively.

Cell suspension cultures: In this experiment and as shown in Fig. 2 calli induced hypocotyl explants were saved in MS liquid fortified with 3 mg L^{-1} 2,4-D in combination with 0.2 mg L^{-1} Kin. The cell number $\times 10^5$ and PCV as growth dynamics was

Table 1: Effect of supplementations MS medium with different concentrations of 2,4-D in combinations with 0.2 mg L^{-1} Kin. on callus formation (%), calli fresh and dry weights induced from hypocotyl, leaf and root explants of *Capsicum annuum*. Cultures were incubated under light conditions 16/8 h at 26±1°C

		Capsicum annuum explants								
Growth regulators		Hypocotyl			Leaf			Root		
Kin (mg L^{-1})	2,4-D (mg L^{-1})	Callus formation (%)	Fresh weight (g/Jar)	Dry weight (g/Jar)	Callus formation (%)	Fresh weight (g/Jar)	Dry weight (g/Jar)	Callus formation (%)	Fresh weight (g/Jar)	Dry weight (g/Jar)
0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	1.0	75.8±3.7	1.43±0.12	0.09±0.005	66.3±4.2	1.27±0.14	0.064±0.001	54.3±3.7	1.19±0.13	0.053±0.0012
0.2	3.0	95.6±8.17	2.25±0.15	0.17±0.007	86.3±5.6	1.89±0.005	0.14±0.007	71.6±4.8	1.35±0.015	0.08±0.004
	5.0	85.4±5.6	1.87±0.15	0.13±0.004	74.5±3.7	1.63±0.15	0.11±0.004	62.8±5.8	1.32±0.16	0.06±0.003

Each treatment is the average of 3 replicates±Standard Error

Fig. 1: Callus and cell suspension production from hypocotyl explants of *Capsicum annuum*

Cultured on MS medium supplemented with 3.0 mg L^{-1} 2,4-D+0.2 mg L^{-1} Kin and incubated under light conditions 16/8 h at 26±1°C

counted and recorded during 4, 8, 12, 16, 20 and 24 days of cultivation. The maximum cell number 4.85×10^5 was recorded after 16 days of cultivation. However, the maximum packed cell volume 1.85 was recorded after 24 days of cultivation. While the significant growth dynamics of PCV was recorded after 16 days of cultivation.

Effect of supplementation modified MS medium with different concentrations of *A. niger* as biotic elicitor on pepper cell number and PCV as growth parameters and capsaicin accumulation rate (mg g^{-1} DW): Pepper hypocotyl cell suspension cultures were cultured on MS medium supplemented with 3 mg L^{-1} 2,4-D and 0.2 mg L^{-1} Kin and elicited with different concentrations (0.0, 0.5, 1, 1.5 and 2%) of suspended *A. niger*. The cell number $\times10^5$ and PCV parameters were recorded after 16 days of cultivation. As shown in Fig. 3 the maximum numbers of cell cultures 6.53, 6.42, 5.76, 5.32 and 4.93×10^5 were recorded with elicited of modified MS medium with 2, 1.5, 1%, 0.5 and 0% (as control

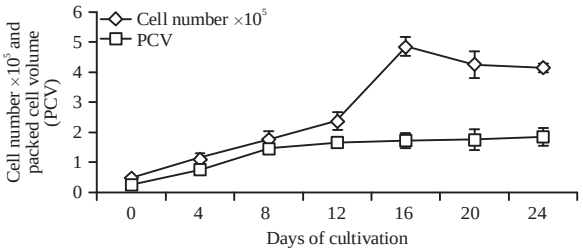

Fig. 2: Effect of number of cultivation days and incubation period on number of cell cultures $\times10^5$ and PCV of pepper hypocotyl cell cultures. Cultures were carried out on MS medium supplemented with 3 mg L^{-1} 2,4-D in combination with 0.2 mg L^{-1} kin

Cultures were incubated under light conditions 16/8 h at 26±1°C, each treatment is the average of 3 replicates ±Standard error

treatment) of *A. niger* as biotic stress, respectively. However, the significant record of cell number $\times10^5$ was recorded with the treatment of hypocotyl cell cultures with 1.5% of extracted *A. niger*. On same situation the highest values of PCV 1.97, 1.95, 1.84, 1.73 and 1.65 were recorded with supplementation of Modified MS medium with 2, 1.5, 1%, 0.5 and 0% (as control treatment) of *A. niger* as biotic stress, respectively. While the significant PCV was recorded with augmented of modified MS medium with 1.5% of *A. niger*.

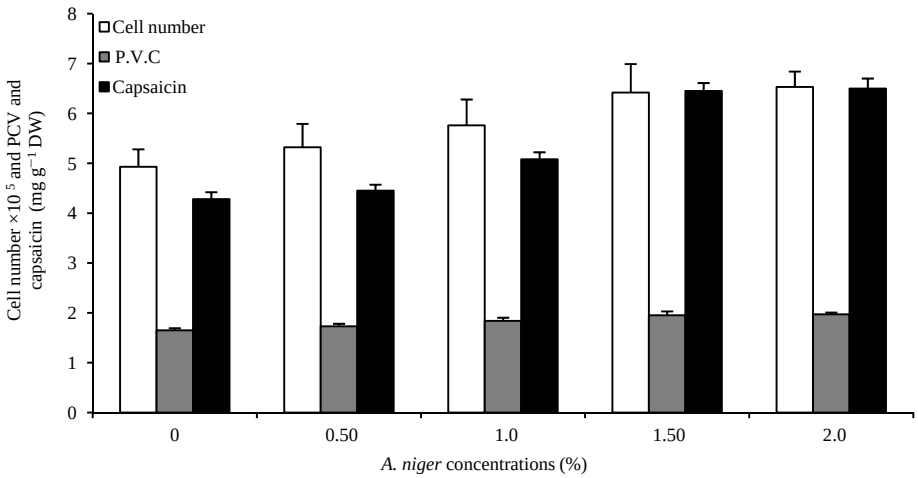

Fig. 3: Effect of supplementation of modified MS medium with different concentrations of *A. niger* (%) as biotic elicitor on pepper cell number and PCV as growth parameters and capsaicin accumulation (mg g^{-1} DW). Cultures were incubated under light conditions 16/8 h at 26±1 °C for 16 days
Each treatment is the average of 3 replicates ±standard error

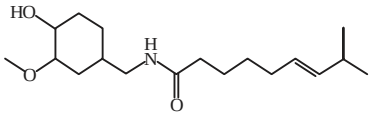

Fig. 4: Capsaicin structure (8-methyl-N-vanillyl-6-nonenamide)

Regarding capsaicin accumulation (mg g^{-1} DW) (Fig. 4), the highest level 6.5 mg g^{-1} DW cell suspension was recorded with supplementation of modified MS medium with 2% of suspended *A. niger.* However, the significant accumulation 6.45 mg g^{-1} DW was recorded with fortified of modified MS medium with 1.5% of *A. niger*.

Effect of supplementation MS medium with different concentrations of methyl jasmonate (µM) as abiotic elicitors on pepper cell number, PCV as growth parameters and capsaicin accumulation (mg g^{-1} DW): Data is illustrated in Fig. 5 clearly showed that the effect of supplementation of modified MS medium with 1.5% of *A. niger* as biotic stress and combined with different concentrations of methyl jasmonate (0.0, 50, 100 and 150 µM) as abiotic elicitor on cell number $\times 10^5$ and PCV as a growth parameters and the accumulation of capsaicin (mg g^{-1} DW). The maximum number of cell cultures 6.18, 6.25 and 5.94×10^5 was recorded with supplementation of cell cultures with 100, 50, 150 µM of methyl jasmonate compared control treatment which recorded 4.95×10^5. However, maximum values of PCV 1.75, 1.64 and 1.57 were recorded with

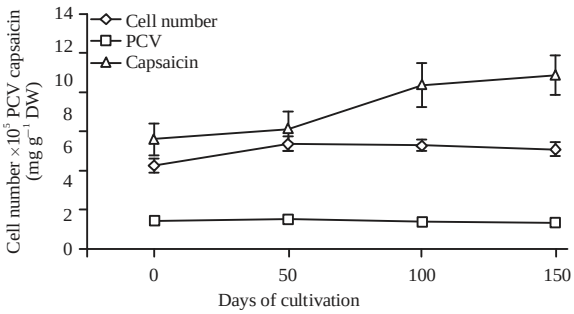

Fig. 5: Effect of supplementation of modified MS medium with different concentrations of methyl jasmonate (µM) as abiotic elicitors in the presence of 1.5% of *A niger* on pepper cell number, PCV as growth parameters and capsaicin accumulation rate (mg/g DW).Cultures were incubated under light conditions 16/8 h at 26±1 °C
Each treatment is the average of 3 replicates ± standard error

supplementation of modified MS medium with 50, 100 and 150 µM of methyl jasmonate compared with control treatment which recorded 1.66. Regarding accumulation of capsaicin in aggregated cell cultures (mg g^{-1} DW) for 16 days, it was found that elicitation of modified MS medium with 100 µM of methyl jasmonate achieved of capsaicin accumulation in significant rate (9.75 mg g^{-1} DW) compared elicitation of cell cultures with 50 or 150 µM of methyl jasmonate which recorded 7.12 and 10.32 mg g^{-1} DW, respectively compared control treatment which provided with 1.5% of *A. niger*.

DISCUSSION

Capsaicin has massive usage in the food, medicine also it is in pharmaceutical industries[9-11]. Further, to obtain high yields suitable secondary products for commercial exploitation; several studies were achieved using plant cell cultures techniques. Some of this targets and efforts have focused on employing precursor feeding, transformation methods and immobilization techniques[33].

Regarding enhancement of capsaicin production in cell cultures using *A. niger* as biotic stress and in agreement of that obtained results, it was reported that metalaxyl induced the phytoalexin capsidiol in the stem of pepper plants which infected with *Phytophthora capsici*[34]. Moreover and as reported in response to fungal infection of *Capsicum annuum,* capsidiol was the major accumulates phytoalexin[35]. Furthermore, capsidiol is identified as biosynthetically derived from the mevalonate pathway via farnesyl pyrophosphate which catalyzed by particular sesquiterpene cyclases[36-38]. In this respect and also as reported that pectinase of *A. niger* and cellulase isolated from *Trichoderma viride* enhanced rapid accumulation of capsidiol in pepper[39]. Moreover and in close of the obtained results, capsidiol production from *in vitro* root cultures of chilli pepper was achieved after treatment with cellulase[23]. In addition, the sesquiterpenoid production from *C. annuum* suspension culture was achieved by applying of cellulase as elicitor[40].

On the same respect and in direction of the effect of methyl jasmonate as abiotic stress on enhancement of capsaicin production in pepper cell cultures, it is well reported that, function of jasmonates on induction of defense has been attributed to the related proteins in plants[41]. It has been postulated that jasmonates might activated of defense gene expression[42-43].

Further and in close of extracted results, the treatment of suspension cell cultures with jasmonic acid or methyl jasmonate, increased the expression of phenylalanine ammonia lyase[44-45]. This may be due to phytoalexin production, as a type of plant self-defense mechanism. As well as, the treatment with methyl jasmonate induces over production of taxane compounds in suspension cultures of taxus[46]. Further, jasmonic acid may be useful to increase the yield of pharmacologically active chemicals and may be used to examine secondary metabolism regulation in plant. Thus and in the same direction and obtained results, it was recommended that elicitor treatment rapidly elevated the cellular level of free linolenic and linoleic acids. It was may be due to the accumulation of jasmonic acid and expression defense genes[47].

In the present investigation, applying of *Aspergillus niger* in combination with methyl jasmonate recorded maximal capsaicin production. Further and in close of the obtained results, the synthesis of secondary metabolites can be enhanced by the addition of several organic compounds to the culture medium[48]. The concept of this study is based on the idea that any compound, which is an intermediate in or at the beginning of a secondary metabolite biosynthetic route, stands a good chance of increasing the yield of the final product. The results presented in this paper it has been possible to identify a number of parallel and contrasting features of the accumulation of capsaicin in cultured cells.

CONCLUSION

The main target of this study is to *in vitro* enhancement the accumulation rate of capsaicin in suspension cultures of *Capsicum annuum* L. In this regards, it was found that fortified of MS medium with 3.0 mg L^{-1} 2,4-D+0.2 mg L^{-1} Kin. gave the highest percentage of both callus and cells production after 16 days of cultivation. On the other hand, It was found that augmentation of MS medium with 1.5% of *A. niger* as biotic in combination with 100 μM of methyl jasmonate as abiotic elicitors achieved of cell growth parameters in parallel with capsaicin production and accumulation in significant rate during 16 days of cultivation.

SIGNIFICANCE STATEMENT

This study discovers the applying of different concentrations of 2,4-D in combinations with Kin. on calli and suspension production. In addition, the optimum concentrations of *Aspergillus niger* and methyl jasmonate as biotic and abiotic stress for enhancement of capsaicin accumulation was reached that can be beneficial for helping the researchers to uncover the critical areas of *in vitro* enhancement and production of capsaicin from suspension cultures of *Capsicum annuum* L. This study will help the researcher to uncover the critical areas of *in vitro* capsaicin production from *C. annuum* L., that many researchers were not able to explore. Thus a new theory on secondary production may be arrived at.

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