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THE EFFECTS OF CELLULASE ON CAPSAICIN PRODUCTION IN FREELY SUSPENDED CELLS AND IMMOBILIZED CELL CULTURES OF *CAPSICUM ANNUUM* L.

CEMIL ISLEK[1*], A.S. USTUN[2] AND E. KOC[2]

[1]*Niğde University, Faculty of Arts and Sciences, Department of Biology, 51200, Niğde, Turkey*
[2]*Ankara University, Faculty of Science, Department of Biology, 06100, Tandoğan, Ankara, Turkey*
[*]*Corresponding author's e-mail: islekcem@hotmail.com*

Abstract

The effect of different concentrations of cellulase on the production of capsaicin in freely suspended cell and immobilized cell cultures of Kahramanmaraş pepper seeds (*Capsicum annuum* L.) were studied. Calluses were obtained from *in vitro* germinated hypocotyl explants of pepper seedlings and cell suspensions were prepared from these calluses. Immobilized cell suspension cultures with calcium alginate and free cell suspension cultures were obtained by using cell suspensions. Elicitor such as cellulase (5-30 µg/ml), was applied both for the free and immobilized cell suspensions and control group without elicitor was prepared. The concentration of capsaicin in freely suspended cells, immobilized cells and their filtrates were identified by HPLC after extraction with ethyl acetate. It was found that the immobilization process had an increasing effect on the capsaicin accumulation. The concentration of capsaicin in the immobilized cells for both control groups and elicitor added samples was higher than the free cells. In general, capsaicin concentration in the filtrate for free cells was higher than the immobilized cells. When all the cellulase and the sampling hours were compared, the highest capsaicin concentration for the immobilized cells was determined as 362,91 µg/g f.w. at the 24th hour for 30 µg/ml cellulase applied samples.

Key words: Capsaicin, Cellulase, Immobilization, *Capsicum annuum*, Plant tissue culture.

Introduction

Capsaicin, which is a component of green pepper fruits (Govindarajan *et al.*, 1987), is derived from phenylpropanoid compounds. Capsaicin and its analogues, called capsaicinoids, are the pungent compounds of the *Capsicum* fruit. Capsaicin, the major pungent compound of hot pepper fruits, is an amide derivation of vanillylamine and 8-methyl non-trans-6-enoic acid (Bennett & Kirby, 1968; Bernal *et al.*, 1993).

Capsaicin is used as food additive and also in medicine as a counter-irritant (Vanisree *et al*., 2004). It is also reported to have antioxidant properties (Sudha & Ravishankar, 2002; Rosa *et al.*, 2002, Ochi *et al.*, 2003; Kogure *et al.*, 2002). Capsaicin has been attributed to have pharmacological effects since ancient times, but its specific applications are yet to be determined including its use in the gastrointestinal tract, for weight-loss and its an analgesic activity (Reyes-Escogido *et al.*, 2011).

Cell cultures of *Capsicum annuum* and *Capsicum frutescens* produce capsaicin and release it to the medium (Johnson *et al.*, 1991). Production of capsaicin is enhanced several fold upon immobilization of cells (Lindsey & Yeoman, 1983).

Elicitation can be an important strategy to improve *in vitro* production of plant secondary metabolites. It has been previously demonstrated that in cell and organ cultures, biotic and abiotic elicitors have effectively stimulated production of almost all chemical classes of plant secondary metabolites (Brooks *et al.*, 1986). The elicitation of capsaicin production under the influence of microbial elicitors has been studied by Johnson *et al.*, (1991) and Sudha & Ravishankar, (2003a, b). Physiologists have intensely studied the action of the highly potent pungency stimuli in Capsicum. The compositional variations, biosynthesis of the functional components, the carotenoids, the volatile and the capsaicinoids are comprehensively reviewed (Govindarajan, 1986). Some researchers have found that exogenous application of cellulase is capable of inducing secondary metabolite formation (Ellialtıoğlu *et al.*, 1998; Ma, 2008). In this study, the influence of immobilization and elicitation of cellulase on the production of capsaicin in the cell suspension culture of *C. annuum* seeds were investigated at different times.

Materials and Methods

Source of plant material: Seeds of *Capsicum annuum* L. were obtained from the Kahramanmaraş Institute of Horticultural Research (Kahramanmaraş, Turkey).

Culture conditions: Seeds of *C. annuum* L. were sterilized with 70% ethanol for 3 minutes and sodium hypochlorite for 20 minutes followed by washing with sterile distilled water.

C. annuum seedlings were germinated in the Murashige & Skoog's (MS) medium (Murashige & Skoog, 1962) without hormone. Hypocotyle explants of seedlings were taken into MS medium (0,1 mg/L kinetin, 1 mg/L 2,4 D, 3% sucrose and 0.7% agar) to produce callus tissue at 25°C. Callus tissues were subcultured two times and taken into liquid medium (MS medium without agar) to produce cell suspensions in 100 ml erlenmeyer flasks. The cultures were incubated on a shaker at 110 rpm and 25°C (Ellialtıoğlu *et al.*, 1998).

Immobilization of cells: One gram of free cells from one month old suspension cultures was separated from the medium by filtration and suspended in a solution of 2, 5% sodium alginate (Johnson *et al*., 1990). Cells suspended in sodium alginate were then extruted into 60 µM calcium chloride dihydrate through sterilized glass pipette affixed to a peristaltic pump.

Elicitor preparation and inoculation: Experiments were carried out in 100 ml erlenmeyer flasks containing 40 ml of fresh medium. Each flask was inoculated with 1g fresh weight of cells. After 14 days of growth, sterile cellulase elicitor was added. Instead of cellulase sterile water was added to control flasks. The cultures were maintained on an orbital shaker at 25°C for 24 h., 48 h. and 72 h. Cells and liquid phase samples were collected after incubation and analyzed for capsaicin. Samples were stored at –70°C until further processing. All the experiments were conducted in triplicate.

Extraction and analysis of capsaicin: For the extraction of capsaicin from suspension cultures, 1 g of cells was ground well with neutralized glass powder (100 mg) using a mortar and pestle, and extracted thrice with 25 ml of ethyl acetate. The extract was centrifuged at 5000 rpm for 15 min and the supernatant was evaporated. The residue was then dissolved in 500 µl of 80% ethyl acetate and used for capsaicin analysis. The procedure was repeated in triplicate to facilitate maximum extraction. Capsaicin from the culture media was extracted thrice with 20 ml of ethyl acetate, each time in a separating funnel. The ethyl acetate layers were pooled and evaporated. The residue was dissolved in 1.0 mL of ethyl acetate for analysis. The extractions were filtered through a 0.45-µm Millipore filter prior to injection on the HPLC (Johnson *et al.*, 1992).

The concentration of capsaicin extracted from the callus and media was estimated by HPLC. The quantification of capsaicin was done on a C18 nucleosil column with detection at 280 nm. The isocratic mobile phase was methanol: water (1% acetic acid) (39: 60 v/v) and a flow rate of 1 ml/min was used.

Result and Discussion

The cellulase elicitor is added to *C. annuum* L., free and immobilized cell suspension cultures as 5, 10, 15 and 30 µg/ml, the capsaicin concentration determined in free and immobilized cell suspension is determined for samples taken at the 24, 48 and 72th hours and results are shown in Tables 1 and 2. Difference in result with regard to time, concentration and method was found statistically significant ($p<0.01$). The Duncan test results of total capsaicin concentrations in free and immobilized cells, in which various concentrations of cellulase is applied and in the filtrate of free and immobilized cells are shown by related averages as well as literal presentation approach.

In free cell samples where cellulase was applied at the 24th hour the change of total capsaicin concentration was found as 73,87-243,3 µg/g fw (Table 1). When the effect of cellulase application on the filtrate of free cells at the 24th hour, it was determined that the capsaicin passing to filtrate increased due to increase of cellulase concentration. In immobilized cell samples where cellulase was applied at the 24th hour the change of total capsaicin concentration was found as 118,8-362,91 µg/g f.w. (Table 2).

When the capsaicin concentration in free cells and immobilized cells after the 24th hour of cellulase application were compared the capsaicin concentration in immobilized cells was determined as two times of the capsaicin concentration in free cells. The highest capsaicin concentration was found at 30 µg/ml cellulase application which is the highest elicitor concentration. The total capsaicin concentration in immobilized cells is higher than the capsaicin concentration in free cells (Fig. 1, Table 2).

Table 1. The effect of cellulase on the accumulation of capsaicin in the free cells.

Time (hour)	Application	Capsaicin (µg/g) t.a.		
		Free cell	Filtrate	Total
24	Control	41,66 ± 0,61 Ad2	32,21 ± 0,82 Ae2	73,87 ± 0,21 Ad2
	5 µg/ml cellulase	87,54 ± 2,72 Ac2	58,5 ± 0,62 Ad1	146,04 ± 2,11 Ac2
	10 µg/ml cellulase	108,8 ± 1,09 Bb2	61,94 ± 0,39 Ac1	170,74 ± 0,71 Bb2
	15 µg/ml cellulase	113,64 ± 5,28 Bb2	73,53 ± 1,51 Bb1	187,18 ± 5,16 Bb2
	30 µg/ml cellulase	154,03 ± 2,63 Aa2	89,27 ± 0,67 Aa1	243,3 ± 3,19 Aa2
48	Control	45,77 ± 0,64 Ac2	33,4 ± 0,51 Ae2	79,17 ± 1,16 Ae2
	5 µg/ml cellulase	57,82 ± 0,84 Bc2	44,32 ± 0,51 Bd1	102,14 ± 0,77 Bd2
	10 µg/ml cellulase	138,42 ± 0,53 Ab2	61,16 ± 0,46 Ab1	199,58 ± 0,60 Ac2
	15 µg/ml cellulase	155,58 ± 1,61 Aab2	71,19 ± 1,20 Bc1	226,77 ± 1,78 Ab2
	30 µg/ml cellulase	165,14 ± 4,30 Aa2	84,00 ± 0,41 Ba1	249,14 ± 3,94 Aa1
72	Control	44,84 ± 0,67 Ab2	33,69 ± 0,71 Ae2	78,53 ± 1,22 Ac2
	5 µg/ml cellulase	47,55 ± 0,80 Bb2	37,22 ± 0,47 Cd1	84,77 ± 0,87 Bc2
	10 µg/ml cellulase	57,35 ± 1,03 Cb2	52,09 ± 1,25 Bc1	109,44 ± 2,08 Cb2
	15 µg/ml cellulase	92,77 ± 1,18 Ca2	76,35 ± 1,23 Ab1	169,12 ± 1,54 Ba2
	30 µg/ml cellulase	85,13 ± 2,69 Ba2	91,27 ± 1,53 Aa1	176,40 ± 4,21 Ba2

Uppercase letters: Vertical column shows the comparison of the same elicitor concentrations at different times,
Small letters: Vertical column shows the comparison of the same elicitor in time
Digits: Horizontal column shows the comparison of the methods according to Duncan test

Table 2. The effect of cellulase on the accumulation of capsaicin in the immobilized cell.

Time (hour)	Application	Capsaicin (µg/g) t.a.		
		Immobilize cell	Filtrate	Total
24	Control	81,59 ± 1,20 Ae1	37,29 ± 0,85 Ab1	118,88 ± 0,37 Ae1
	5 µg/ml cellulase	161,66 ± 1,97 Bd1	41,19 ± 0,20 Ba2	202,84 ± 2,12 Bd1
	10 µg/ml cellulase	245,93 ± 3,30 Bc1	40,26 ± 0,82 Ba2	286,18 ± 3,22 Bc1
	15 µg/ml cellulase	288,84 ± 4,37 Ab1	40,55 ± 0,47 Ca2	329,39 ± 4,07 Ab1
	30 µg/ml cellulase	322,52 ± 4,05 Aa1	40,39 ± 0,71 Ba2	362,91 ± 4,54 Aa1
48	Control	82,02 ± 1,28 Ae1	37,69 ± 1,04 Ad1	119,71 ± 1,85 Ad1
	5 µg/ml cellulase	197,31 ± 1,10 Ac1	42,51 ± 0,79 Ac1	239,81 ± 1,83 Ac1
	10 µg/ml cellulase	276,64 ± 1,79 Aa1	39,00 ± 0,46 Bd2	315,64 ± 2,25 Aa1
	15 µg/ml cellulase	242,97 ± 2,33 Bb1	48,54 ± 2,00 Bb2	291,51 ± 0,51 Bb1
	30 µg/ml cellulase	175,5 ± 1,69 Bd1	55,17 ± 0,28 Aa2	230,67 ± 1,70 Bc1
72	Control	79,53 ± 1,48 Be1	36,66 ± 0,17 Ad1	116,19 ± 1,63 Ae1
	5 µg/ml cellulase	191,43 ± 2,49 Ac1	39,22 ± 0,01 Bc1	230,66 ± 2,50 Ac1
	10 µg/ml cellulase	272,84 ± 3,38 Aa1	43,92 ± 0,17 Ab2	316,76 ± 3,31 Aa1
	15 µg/ml cellulase	215,06 ± 1,84 Cb1	55,99 ± 0,45 Aa2	271,05 ± 2,15 Cb1
	30 µg/ml cellulase	170,24 ± 1,30 Bd1	42,42 ± 0,36 Bb2	212,66 ± 1,00 Bd1

Uppercase letters: Vertical column shows the comparison of the same elicitor concentrations at different times,
Small letters: Vertical column shows the comparison of the same elicitor in time
Digits: Horizontal column shows the comparison of the methods according to Duncan test

In free cell samples where cellulase was applied at the 48th hour the change of total capsaicin concentration was found as 79,17-249,14 µg/g f.w. (Table 1). At the 48th hour, highest capsaicin concentration was found at 30 µg/ml cellulase application in free cell. Except 5 µg/ml cellulase application, in other application concentrations more capsaicin accumulation compared with other control samples was determined in free cell ($p<0,01$). Highest capsaicin concentration passing to filtrate at the end of cellulase application was determined at 30 µg/ml concentration (Fig. 2). The capsaicin concentrations in the filtrates of free cells at the 48th hour increased due to concentration increase ($p<0,01$). In immobilized cell samples where cellulase was applied at the 48th hour the change of total capsaicin concentration was found as 119,71-315,64 µg/g f.w. (Table 2). Highest capsaicin accumulation in immobilized cells at the 48th hour of elicitation was determined at 10 µg/ml cellulase application ($p<0,01$).

In free cell samples where cellulase was applied at the 72th hour the change of total capsaicin concentration was found as 78,53-176,40 µg/g fw (Table 1). At the 72th hour, highest capsaicin concentration in free cells was found at 15 µg/ml cellulase application (Fig. 3). In immobilized cell samples where cellulase was applied at the 72th hour the change of total capsaicin concentration was found as 116,19-316,76 µg/g fw. (Table 2). For immobilized cells, highest capsaicin concentration in free cells was found at 10 µg/ml cellulase application at the 72th hour ($p<0,01$).

When the capsaicin concentrations forming in free cells in different times are compared, the difference between the average concentration is not statistically significant.

At 30 µg/ml application concentration of cellulase and at the 24th hour and 48, the capsaicin concentrations were found to be 3 times of the control while at the 72th hour the capsaicin concentration was half of the control. This decrease was found statistically significant ($p<0,01$) The capsaicin concentrations at 5 µg/ml application concentration of cellulase and at the 48th hour and 72, no significant difference was found between the capsaicin concentrations (Tables 1-2, Figs. 2-3).

When the capsaicin concentrations forming at different times in immobilized cell control groups were compared according to Duncan test, the decrease in capsaicin concentration at the 72th hour was found significant. ($p<0,01$).

At this time period, the highest capsaicin was found at 30 µg/ml cellulase application and lowest was found in control group (Fig. 3). The effect of cellulase concentrations on capsaicin concentration at the 72th hour was found statistically significant. ($p<0,01$).

The capsaicin concentration in free cell filtrate was found more than the capsaicin concentration in immobilized cell filtrates. This difference is statistically significant ($p<0,01$). When the capsaicin concentrations in free and immobilized cell filtrate control samples were compared the capsaicin concentration in immobilized cells were found higher.

In free cell samples where cellulase was applied at the 72th hour the change of total capsaicin concentration was found as 78,53-176,40 µg/g f.w. (Table 1). In these cells, the difference between control application and 5 µg/ml cellulase application concentrations is not statistically significant. The highest increase in capsaicin concentrations was determined at 30 µg/ml cellulase concentration. There is no statistical difference between the obtained result and the concentration obtained at 15 µg/ml cellulase concentration. In immobilized cells, the highest increase in capsaicin concentrations at the 72th hour was determined at 15 µg/ml cellulase concentration (Fig. 3). In total immobilized cells at the 72th hour, the effect of control application and elicitor concentrations on capsaicin concentration is statistically significant.

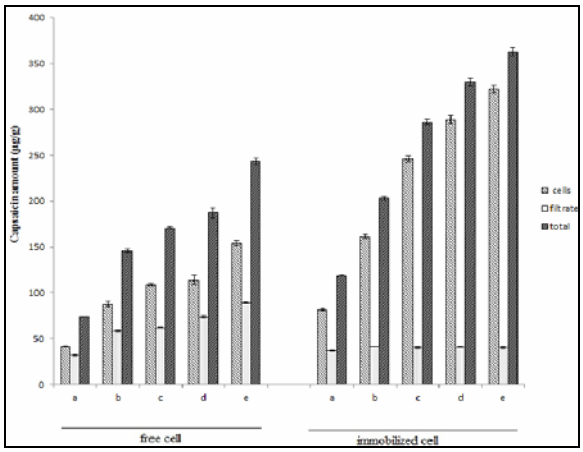

Fig. 1. The effect of different concentration of celluase on the capsaicin concentration in the free and immobilized cells at hour 24
a: Control, b: 5 µg/ml cellulase , c: 10 µg/ml cellulase , d: 15 µg/ml cellulase , e: 30 µg/ml cellulose

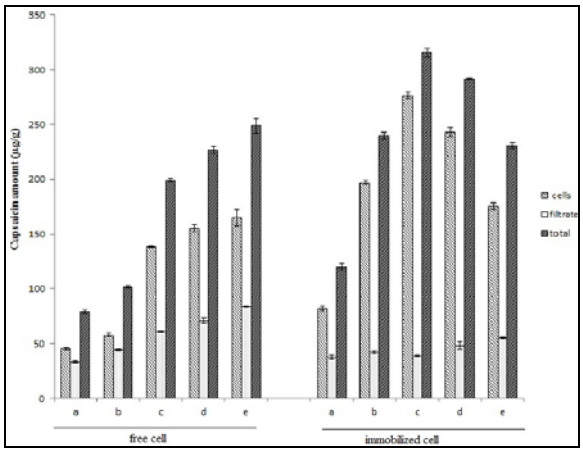

Fig. 2.The effect of different concentration of celluase on the capsaicin concentration in the free and immobilized cells at the 48th hour
a: Control b: 5 µg/ml cellulase c: 10 µg/ml cellulase d: 15 µg/ml cellulase e: 30 µg/ml cellulase

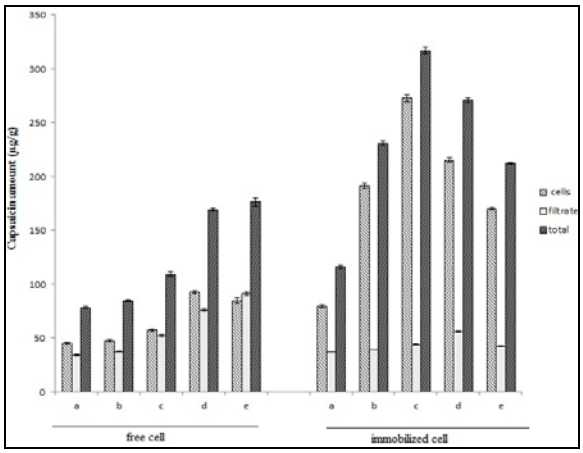

Fig. 3.The effect of different concentration of celluase on the capsaicin concentration in the free and immobilized cells at the 72th hour
a: Control b: 5 µg/ml cellulase c: 10 µg/ml cellulase d: 15 µg/ml cellulase e: 30 µg/ml cellulase

When the capsaicin concentrations forming at different times in immobilized cell control groups were compared statistically, the difference between averages was not found statistically significant. In immobilized cells, at the 24th hour, for 5 µg/ml and 10 µg/ml cellulase applications, total cellulase accumulation increases. Capsaicin concentration decreases as the elicitation period increases. At 30 µg/ml cellulase concentration, capsaicin concentration decreases as the elicitation period increases. This decrease is statistically significant ($p<0,01$) In terms of total capsaicin concentrations, cellulase application was more successful in immobilized cells at 24th hours. In immobilized cells, highest capsaicin concentration was determined at the 24th hour for 30 µg/ml cellulase application (Table 2).

Previous studies showed that the immobilized cell cultures of *Capsicum* spp., produces a few times more capsaicin compared with free suspension cultures. The immobilization process in our study was found to be effective on capsaicin concentration for all cellulase concentrations and elicitation periods. The obtained results are in accordance with immobilization studies which were previously conducted (Lindsey & Yeoman, 1983; Lindsey, 1985; Johnson *et al.*, 1991).

In a study conducted regarding alkaloid accumulation in cell cultures it was demonstrated that the structural organization and growth rate of cells were found to be closely related with secondary metabolic function activity and had determining effect on the occurring reactions (Lindsey & Yeoman, 1983). It was also determined that cells that structurally stick together synthesize highest concentration of alkaloid and that the metabolic pioneers that are used in both primary and secondary metabolic functions are not used in protein synthesis but instead in secondary metabolic reactions such as synthesis of phenolic compounds.

There are no studies regarding cellulase elicitor effect on capsaicin synthesis. Cellulase elicitor stimulate phythoalexin formation in pepper like in most plant types. It was determined that cellulase has an increasing effect for capsidiol (a pepper phythoalexin) in *Capsicum annuum* callus suspension culture (Ellialtıoğlu *et al.*, 1998). The effective elicitor concentration and time in free cells was found as 30 µg/ml and the 24-48th hours in our study (Tables 1-2). The cells that are immobilized in culture environment and that are located structurally together grow relatively slower than the cells in culture environment so their primary metabolic activities are lower and therefore have more capacity to synthesize secondary metabolic compounds.

Conclusions

As a result of this study, it was found out that the capsaicin concentration in cells was more than filtrates. We opine consider that cellulase elicitor directly contacts free cells and effect cell membranes and therefore forces the capsaicin to pass to the filtrate. In immobilized cells, calcium alginate layer is considered to relatively decrease the passing of cellulase to filtrate, compared with free cells. All cellulase concentrations applied in immobilized cells was effective on capsaicin production in our study.

The most effective elicitor concentration and application time was found to be 30 µg/ml cellulase and 24 hours. At 48 and 72 hours for 30 µg/ml cellulase concentrations, a decrease in capsaicin concentrations was observed compared to the level in 24 hours. When elicitor is applied to the culture at high concentrations and long time period, the culture is harmed or not affected at all. There must be an optimum value where maximum capsaisin concentration is formed for cellulase concentration and elicitation period. As a matter of fact, while elicitor period increases in immobilized cells for 5 and 10 µg/ml cellulase application, elicitation period decreases for 15 and 30 µg/ml cellulase application. Elicitors can be used to induce capsaicin accumulation in cultured plant cells**.** There is a need for screening a number of possible biotic and abiotic elicitors for their effect on capsaicin synthesis before applying them in industrial scale.

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