Effect of Light, Temperature, and Shaking Speed on Production of Capsaicin in Suspension-Cultured Jalapeno Pepper (*Capsicum annuum* L.)

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Capsaicin synthesis by suspension cultured cells of Jalapeno pepper (*Capcicum annuum* L.) was assessed *in vitro* under various conditions including temperature (23 and 30°C), light intensity (with light and without light), and shaking speed (110 and 200 rpm). Capsaicin production increased, while the cell biomass growth decreased possibly due to the production of a secondary metabolite. Capsaicin synthesis was primarily affected by light condition. Cells cultivated at 110 rpm and 23°C under light condition yielded the highest fresh weight, while those cultivated under the same condition, but without light resulted in the lowest cell mass. Capsaicin content in cells of 18-day-old pepper grown at 110 rpm and 23°C under light was 0.125% of the cell mass. However, without light treatment, the capsaicin content in cells at the same shaking speed and temperature increased up to 169%, indicating no light is favored in the capsaicin synthesis by Jalapeno pepper. Increasing the shaking speed from 110 to 200 rpm without light enhanced the capsaicin synthesis. Results of this study demonstrate that light condition is the limiting factor in the synthesis of capsaicin in tissue-cultured Jalapeno pepper cells.

Key words: *pepper, suspension-cultured-cells, capsaicin synthesis, physical stress.*

Pungency, one of the most prominent characteristics of Jalapeno pepper (*Capsicum annuum* L.), results from the accumulation of natural secondary metabolite capsaicin in the fruit, which is known to affect the nerve sensors of the human mouth to impart the sensation of heat.[1-3]

Capsaicin was discovered by Thresh in 1876 and its chemical structure was identified as vanillylamide of isodecenoic acid by Nelson in 1919.[4] After the discovery of its chemical structure, capsaicin had been widely used in many different areas. Capsaicin is known to have a broad spectrum of activity, including the inhibition of bacterial multiplication, germination of fungal spores, and multiplication of cancer cells in tissue cultures.[3,5-7] Recently, pain-relieving activity in human body has also been reported.[8]

Usefulness of capsaicin in pharmaceutical and food industries have brought about attempts to increase the amount of capsaicin in cells of pepper fruit *in vitro*. Production of the secondary metabolite increased by feeding precursors in phenylpropanoid biosynthesis, stimulating with a fungal culture extract, and/or limiting nutrient to the cells.[9] In tissue cultures, however, the amount of capsaicin accumulated in the cells is still too low.[10]

Extracellular accumulation of capsaicin is influenced by the chemical environment and light. Light is important for capsaicin production since it is required for the synthesis of long chain fatty acids from ATP, CoA, and acetate.[11,12] Capsaicin synthesis is known to be inversely related with growth rate, possibly due to the competition between protein and phenylpropanoid syntheses for the common primary metabolite phenylalanine.[10,13]

Increasing the production of capsaicin on a factory scale would be a challenge to the pharmaceutical and food industries. However, optimization of the laboratory-scale fermentation should first proceed. The objective of this research was thus to optimize the growth conditions for in vitro production of capsaicin from tissue-cultured cells of Jalapeno pepper (*Capsicum annuum* L.).

Materials and Methods

Jalapeno pepper callus and suspension culture. Jalapeno M (*Capsicum annuum* L.), obtained from the Texas A & M Agricultural Research and Extension Center (Weslaco, TX, U.S.A.), was used as a source of callus initiation. Seeds were surface-disinfected for 10 min in a 5% hypochlorite solution containing 1 or 2 drops of Tween 20, washed with sterile water twice, and placed on two layers of water flooding sterile filter paper (MFS No. 5A) in petri dishes. They were incubated at 25°C under 30 to 60 $\mu E \cdot m^{-2} \cdot s^{-1}$ cool white fluorescent light (Duro-Test Co., NJ 07047, USA). Two weeks after incubation, the hypocotyls were excised and transferred on Murashige and Skoog (MS) medium (Sigma M-5519) con-

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taining 1% sucrose, 0.3 mg · l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.1 mg · l^{-1} kinetin. To establish a suspension culture, 1~2 g fresh weight callus was transferred into 250 m*l* triple baffled Erlenmeyer flasks containing 60 m*l* of MS medium. Flasks were agitated at 110 rpm on an orbital shaker at room temperature (23°C) under 50 to 60 μE · m^{-2} · s^{-1} vital fluorescent light (Duro-Test Co., NJ 07047, USA). Vigorously growing suspension cultures were established in the modified MS medium, provided with plant growth regulators, naphthalylacetic acid (NAA) and 6-benzyamino purine (BAP).

Analysis of capsaicin production. capsaicin production of vigorously growing suspension culture was monitored. Suspension culture cells were separated from the culture medium by vacuum filtration using Whatman No. 44 filter paper in a Büchner funnel. Cultured filtrate was extracted twice with two volumes of chloroform in separatory funnels. The extracts were dried over sodium sulfate and evaporated to dryness *in vacuo* (130 rpm, 45°C). The residue suspended in methanol was applied to high-performance liquid chromatograph (HPLC: Beckman, System Gold) employing a 5-micron ODS spherical C18 analytical column (4.6 mm width; 250 mm length; Bodman Chemicals). Absorbance profiles for chloroform extracts of culture filtrates was 280 nm. Mobile phase was methanol/water (60/40, v/v) at a flow rate of 1.5 m*l* · min^{-1}. Control was a pure capsaicin obtained from Sigma (#5918, St. Louis, MO, USA) dissolved in methanol/water (60/40, v/v).

Treatments and statistical analysis. Treatments of temperature (23 and 30°C), light (with and without light), and shaking speed (110 and 200 rpm) were included to assess their effects on the capsaicin production. Means of three replicates for determination of callus fresh weight at each sampling time were analyzed with single-degree-of-freedom orthogonal contrasts. Slopes of treatment regressions over time were separated by heterogeneity test of slopes. Significance of treatments on the capsaicin productions of the 18-day-old cultured media was determined using Duncan's multiple range test. Statistical values were obtained using the general linear model and analyses of variance procedure of SAS (SAS Institute Inc., Cary, NC 27511, USA). Main effects of treatments were judged significant at $P \leq 0.05$.

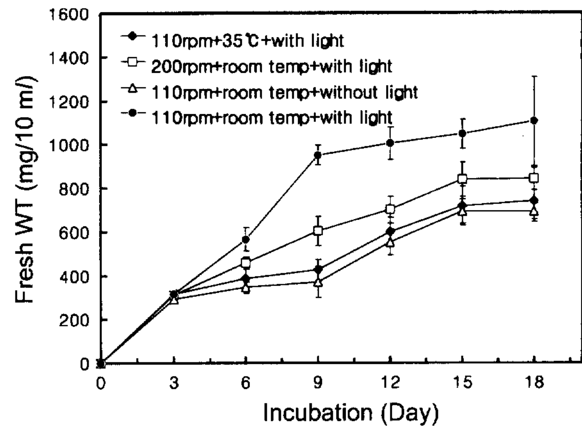

Fig. 1. Effect of shaking speed, light, and temperature on the growth of tissue-cultured Jalapeno pepper (*Capsicum annuum* L.) cells in Murashige and Skoog medium. The cells in triple baffled Erlenmeyer flasks were agitated at 110 or 200 rpm on an orbital shaker at room temperature (23°C) or 35°C, with or without 50 to 60 μE m^{-2} · s^{-1} vital fluorescent light. Bars indicate standard deviation.

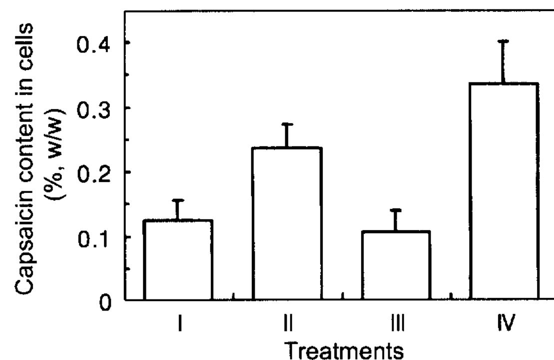

Fig. 2. Capsaicin production of 18-day-old tissue-cultured Jalapeno pepper (*Capsicum annuum* L.) cells. Treatments shown in bars from left to right indicate I [110 rpm + room temperature (23°C) + with light], II [110 rpm + room temperature + without light], III [110 rpm + 35°C + with light], and IV [200 rpm + room temperature + with light], respectively. Capsaicin production from chloroform-extracted pepper tissue cultured medium was analyzed via HPLC employing a C18 reverse-phased column at 280 nm. Mobile phase was methanol/water (60/40, v/v) at a flow rate of 1.5 m*l* · min^{-1}. Bars indicate standard deviation.

Results and Discussion

Incubation time, agitation speed, incubation temperature and light affected the growth of the tissue-cultured cells of Jalapeno M (Fig. 1). Fresh weight of cells in the medium at 110 rpm and 23°C under light condition resulted in the highest cell mass compared to other treatment combinations. The fresh weight of cells at the condition of 110 rpm and 23°C, without light, on the other hand, showed the lowest cell mass. These results indicate that light is an important factor for the cell growth of Jalapeno M. In fact, light is needed for the production of energy and the activation of enzymes, which are involved in several physiological and biochemical processes.

Under conditions of 23°C without light, the shaking speed was the determinant for the cell growth (Fig. 1). Fresh weight of cells at 110 rpm was higher than that at 200 rpm (110.6 mg · ml^{-1} vs 69.4 mg · ml^{-1}), suggesting that higher shaking speed reduced the cell growth significantly ($p<0.05$).

Temperature affected the cell growth of Jalapeno M (Fig. 1). At the conditions of 110 rpm and with light, fresh weight of cells at 23°C was 1106 mg/10 m*l*, while that at 30°C was 74.2 mg · ml^{-1}. These results indicate that room temperature was favored for the cell growth of Jalapeno M.

Capsaicin production by the pepper cells in the media was mainly affected by the light availability and shaking speed (Fig. 2). Capsaicin content in cells of 18-day-old media at 110

rpm and 23°C under light condition was equivalent to 0.125% of the cell mass. Cells grown at 200 rpm and 23°C with light contained 88% more capsaicin than the cells grown at 110 rpm and 23°C with light ($P \leq 0.05$), while the cell growth was retarded at a higher agitation speed. These results revealed that the cell growth was inversely correlated with the capsaicin production. At present, it is not clear why cells produce more capsaicin at a higher shaking speed. One possible explanation is that the shaking speed activates the physiological and biochemical processes in cells. Further researches are required to determine the growth, and physiological and biochemical responses of cells at higher shaking speeds than 200 rpm.

Cells grown at 110 rpm and 23°C without light contained 169% more capsaicin than the cells grown under the same condition, but with light ($P \leq 0.05$). However, temperature did not significantly affect the production of capsaicin in the cultured cells ($P = 0.05$). Our results showed that light is the limiting factor for capsaicin production. Light is required, in fact, for the synthesis of long chain fatty acids from ATP, CoA, and acetate.[11] It is interesting to note in this study that the growth of Jalapeno M cells increased with light availability, but the production of capsaicin increased significantly by blocking the light source. This implies that pepper plants might be more pungent when they are grown under shade.

The overall results of this study showed that the capsaicin synthesis is affected by various physical stresses. To expand the results of this research to the industrial-scale production of capsaicin, however, optimization of the organic compounds in the media should proceed first to provide an ample amount of the capsaicin precursors. Furthermore, studying the relationship of growth condition of the pepper cells and the production of capsaicin in time sequence should also be performed.

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