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Research Article

Silver nanoparticles for the enhancement of accumulation of capsaicin in suspension culture of *Capsicum* sp.

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ABSTRACT

Secondary metabolites can be very important chemicals for the development of plants. The secondary metabolite in this study is obtained from *Capsicum*. Capsaicin is industrially very important due to its wide use range. The study involved is the use of silver nanoparticles for elicitation of levels of capsaicin. Mediums with different hormonal combinations were prepared and growth was studied using qualitative analysis using iodine fumes, Gibbs Reagent and quantitative analysis using phosphomolybdic acid method and Folin–Ciocalteu method. This brings down a conclusion that the nanoparticles acted as an elicitor and brought about capsaicin increase effectively. Extraction of the metabolite from these natural sources also proves easier and the product can be obtained in a much purified form.

KEY WORDS: *Capsicum*, capsaisin, elicitor, secondary metabolite, silver nanoparticles

INTRODUCTION

Secondary metabolites act as an important chemical product for the development of plants (Croteau *et al.*, 2000; Balandrin *et al.*, 1985). One such important plant secondary metabolite is obtained from the genus *Capsicum*. *Capsicum* is known to encompass a wide variety of secondary metabolites namely ascorbic acid (vitamin C), carotenoids (provitamin A, tocopherols (vitamin E), flavonoids, and capsaicinoids (Heike and Knorr, 1995; Rao and Ravishankar, 2002; Bouvier *et al.*, 2003). The capsaicinoids include capsaicin, dihydrocapsaicin, nordihydrocapsaicin, homodihydrocapsaicin, homocapsaicin, and non-ivamide. Capsaicin and dihydrocapsaicin account for approximately 90% of capsaicinoids in chili pepper fruit, are the two most potent capsaicinoids and their molecules differ only in the saturation of the acyl group (Reyes-Escogido Mde *et al.*, 2011). Capsaicin can be commercially important.

The burning sensation caused by capsaicin on coming in contact with the mucous membranes has caused it to become a popular additive in food products requiring a heat or a spicy taste (Duarte *et al.*, 2000). Although capsaicinoids have long been associated to the food industry, recent applications have been extended to other fields. Due to a decrease of sensitivity of skin or mucous membranes after repeated contact with capsaicinoids, they are now used for the treatment of rheumatic and some other pain-causing affections (Govindarajan and Sathyanarayana, 1991; Markovits and Gilhar, 1997). Application of capsaicin cream can be in chronic distal painful polyneuropathy (Low *et al.*, 1995). There is a wide range of capsaicin application in clinical and medical industry (Epstein and Marcoe, 1994; Petsche *et al.*, 1983; Bernstein *et al.*, 1987). Hence, this topic is very important for studies.

Plant tissue culture techniques have been used to bring about the production of a number of metabolites of medicinal importance. The callus and cell cultures suspended in a liquid media are treated to desired environmental conditions aseptically to bring about the production of the desired metabolite sometimes, elicitors are used. An "elicitor" may be defined as a substance which when introduced in small concentrations to a living cell system, initiates or improves the biosynthesis of specific compounds. Elicitation is the induced or enhanced biosynthesis of metabolites due to the addition of trace

amounts of elicitors (Namdeo, 2007; Balandrin *et al*., 1985).

In this current project, the production of capsaicin was done by growing callus cultures from chili explants on a suitable Murashige and Skoog (MS) defined medium. Once of considerable size, they calli were then transferred to the same MS (Murashige and Skoog, 1962) defined liquid medium to be kept on a shaker for a designated number of days. Each flask suspended with the callus was treated to a 50 ppm solution of silver nanoparticles that was used as an elicitor to bring about elicitation in the levels of capsaicin.

MATERIALS AND METHODS

Media for Callus Culture

The explant taken was from *Capsicum frutescens*. The growth medium taken into consideration was MS basal medium (defined). Four types of media were used with different plant growth hormones to test the best suited medium for the growth of the explant into a callus. Appropriate amounts of the stock solutions are added to distilled water in a separate flask to make the volume to 1 L. Each stock solution is made up of varying concentrations of salts and nutrients. These varying concentrations bring about the growth of the callus. A detailed account of the nutrients is enlisted below.

Macronutrients (×20) consisted of ammonium nitrate - 1650 mg/L; calcium chloride - 440 mg/L; manganese sulfate - 370 mg/L; potassium nitrate - 1900 mg/L; potassium phosphate - 170 mg/L. Micronutrients (×100) as boric acid - 6.2 mg/L; cobalt chloride - 0.03 mg/L; cupric sulfate - 0.03 mg/L; manganese sulfate - 22.3 mg/L; potassium iodide - 0.83 mg/L; sodium molybdate - 0.25 mg/L; zinc sulfate - 8.6 mg/L. Vitamins (×100) glycine - 2 mg/L; inositol - 100 mg/L; nicotinic acid/niacin - 0.5 mg/L; pyridoxine HCl - 0.5 mg/L; thiamine HCl - 0.1 mg/L. Iron source ×200 as ferrous sulfate - 27.8 mg/L; Na_2 ethylenediaminetetraacetic acid, 37.2 mg/L. The pH of the medium is set in between pH 5.6 and 5.8. Solidifying the medium for slant preparation is done by adding 1% agar and is added only after the pH is set. For defined media, plant growth hormones are added in fixed concentrations taking into consideration the explant and also the callus growth requirement, i.e., rooting or shooting. In the current experiment, four sets of defined media were prepared. The hormones are added using the above formula, from a stock having concentration of 1 mg/10 ml. The hormones are added post adjustment of pH and before the addition of agar.

Medium with Different Hormonal Combinations and Concentrations

1. MS (basal) + 5 mg/L 6-benzylaminopurine (BA) + 0.1 mg/L α-naphthaleneacetic acid (NAA)
2. MS (basal) + 5 mg/L adenine sulfate + 0.1 mg/L NAA
3. MS (basal) + 5 mg/L BA + 3 mg/L $AgNO_3$ (nanoparticles)
4. MS (basal) + 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) + 1 mg/L kinetin.

About 60 ml (4 tubes) of the above defined media was prepared and was used as a slant for the inoculation of the explant. The leaf, shoot, node/internode of the explant was placed aseptically in 3 of the tubes. In the last tube, the placenta along with one-two seeds from the bell pepper fruit was inoculated.

Growth Studies for the Callus

All the inoculated tubes were checked on a daily bases for the contamination and for callusing. Once the callus had grown to a desire size, 0.3-0.5 g of the callus was weighed and suspended in 40 ml of the liquid media in 3 flasks each. The flasks were kept for 3 days, 6 days, and 9 days on a shaker. To each flask, 5 ml of silver nanoparticle solution was added, i.e. to give a 50 ppm concentration of nanoparticles, before placing on the shaker. A control flask was kept with only callus suspended in liquid medium without nanoparticles. The experiment was carried out in duplicates. The callus was then used to conduct tests as per the quantitative and qualitative estimations for capsaicin content.

Silver Nanoparticles Solution Preparation

About 5 g of plant extract was cut into small pieces and boiled with 50 ml of deionized water for 10 min. The solution was filtered through muslin cloth and centrifuged. The filtrate was used as the reducing agent for the preparation of silver nanoparticles. About 1 g of plant extract was added to 9 ml of 1 mM silver nitrate solution and kept at room temperature. The color change from yellow to brown indicates the reduction of pure Ag^+ ions to silver metal nanoparticles.

Methods

Qualitative estimation of capsaicin

Qualitative estimation of capsaicin was conducted using liquid thin layer chromatography (TLC). The chromatography was conducted on both silica gel coated over aluminum and also silica gel coated over a glass slide. Reagents used were methanol, chloroform, acetic acid, Gibb's reagent (2,6-dichloroquinone-4chloroimide),

iodine fumes, ammonia fumes, chloroform:acetic acid:methanol as 9.5 ml:0.5 ml:0.1 ml (volume basis).

Extract Preparation

The samples used were fresh *Capsicum* fruit, *Capsicum annum*, *C. frutescens*, callus from the liquid medium for day 6 and day 9. About 1 g each of the three samples is taken and crushed in 5 ml of methanol. The *C. annum* and *C. frutescans* being dry samples are initially allowed to soak in methanol till the methanol starts acquiring some color. The extract is then refluxed for 10 min. The refluxed sample is then collected and allowed to cool and reconstituted in 5 ml methanol. About 1 ml out of the extract is taken in a petri plate and allowed to evaporate till it gets concentrated. The above sample further was subjected to TLC. TLC chamber was spree saturated with the mobile phase and the plates once loaded are then placed in the chamber and allowed to run till about 3/4th the length of the plate.

Before the drying begins, the solvent front is marked lightly at one side of the plate.

Once the solvent has run the plates are removed and air dried till the solvent traces have been lost completely. Two methods were further used and R_f is the retention factor.

Using iodine fumes

As the solvent system is running, in another chamber, some iodine crystals were taken and the chamber was covered with a Petri plate and allowed to saturate. After the chromatogram on the aluminum plate was dried, it was immediately placed in the iodine fumes saturated chamber to bring about the development of the capsaicin spot that appeared brown in color. The plate was then taken and used for calculating the retention factor of R_f value.

Using Gibb's reagent

Another solvent system that was used is the Gibb's reagent, also known as 2,6-dichloroquinone-4chloroimide. It was prepared by dissolving 0.5 g of the reagent in 100 ml of methanol. The plate once it run was dipped in the Gibb's reagent and then let to dry. As the reagent was drying, another chamber was simultaneously set up containing ammonia fumes. Once the reagent had dried, the plate was then exposed to the ammonia fumes to bring about the development of the spot.

Quantitative estimation of capsaicin

Phosphomolybdic acid, 20.4% NaOH, methanol were used.

Phosphomolybdic acid method (Singleton and Rossi, 1965)

About 1 g of material was taken and extracted in 5 ml of methanol. In case of dry material like chili, the material was first allowed to soak till the methanol acquired color. The material was then refluxed for 10 min. After cooling, the sample was reconstituted in 5 ml of methanol. The sample was then centrifuged and the supernatant was used for the following test. Bismuth nitrate was prepared in the concentrations of 0.2, 0.4, 0.6, 0.8, and 1 and the volume made up to 1 ml using D/W. The tubes were treated to the following chemicals and optical density (OD) taken at 650 nm to provide a standard graph for quantitative estimation of capsaicin. About 1 ml of the sample extract of the supernatant is allowed to evaporate. To the evaporated sample, 2 ml of 0.4% NaOH is added and mixed thoroughly. About 6 ml of phosphomolybdic acid is added to the above sample and is mixed and kept aside for 1 h. The OD is then read at 650 nm. In case if the solution is dark in color, it is diluted before taking the reading.

Quantitative estimation of the total phenolic content

Reagents used were methanol, Folin's–Ciocaltu reagent (dilute the Folin's reagent by taking 0.5 ml of the original solution and diluting it to 5 ml with distilled water), 7.5% Na_2CO_3, 0.01 g of gallic acid dissolved in 10 ml of D/W.

Folin's–Ciocalteu method

About 1 g of material was taken and extracted in 5 ml of methanol. In case of dry material like chili, the material was first allowed to soak till the methanol acquired color. The material was then refluxed for 10 min. After cooling, the sample was reconstituted in 5 ml of methanol. The sample was then centrifuged and the supernatant was used for the following test. To 1 ml of the diluted Folin's reagent, add 2 ml of 7.5% Na_2CO_3. To the above mixture, add 1 ml of the plant extract prepared. Various concentrations of Gallic acid was prepared i.e. 0.2, 0.4, 0.6, 0.8, and 1. The volume was made up to 1 ml using D/W. This was used as the standard for the quantitative estimation of phenols. About 1 ml of diluted Folin's reagent was added to each tube, to which 2 ml of 7.5% Na_2CO_3 was added. All the tubes were vortexed for about 10 s. They were then kept in a warm water bath for 15 min. The OD was taken at 765 nm.

RESULTS

Callus extract control is used for comparison between use of silver nanoparticles and control culture. Control culture is without the use of nanoparticles.

Growth Results Interpretation for Callus

Among the four media, MS media with different hormonal combinations and concentration used for the growth of the callus, the media MS (basal) + 2 mg/L 2,4-D + 1 mg/L kinetin, it was proved to give the best for the formation of a callus within a short period of time (2 weeks). All the explants showed immediate callusing; however, the placenta and seed inoculation in all of the four media did not show any kind of swelling or initiation of callusing. All the results are shown in Tables 1-4.

Table 1: Results for MS (basal)+5 mg/L BA+0.1 mg/L NAA

Study parameter	Day 1	Day 7	Day 14
Shoot	-	-	-
Internode	Swelling (no contamination)	Swelling persists (no contamination)	-
Leaf	Swelling (no contamination)	Swelling persists (no contamination)	-
Placenta	Very slight swelling (no contamination)	No change (no contamination)	No change observed (No contamination)

MS: Murashige and Skoog, BA: 6-benzylaminopurine, NAA: α-naphthaleneacetic acid

Table 2: MS (basal)+5 mg/L Ads+0.1 mg/L NAA

Study parameter	Day 1	Day 7	Day 14
Shoot	-	-	-
Internode	Swelling (no contamination)	Swelling persists (no contamination)	-
Leaf	Swelling (no contamination)	Swelling persists (no contamination)	Tube showed contamination
Placenta	Very slight swelling (no contamination)	No change (no contamination)	No change observed (no contamination)

MS: Murashige and Skoog, NAA: α-naphthaleneacetic acid, Ads: Adenine sulfate

Table 3: MS (basal)+5 mg/L BA+3 mg/L $AgNO_3$ (nanoparticles)

Study parameter	Day 1	Day 7	Day 14
Shoot	-	-	-
Internode	Slight swelling (no contamination)	No visible change (no contamination)	Tube showed contamination
Leaf	Slight swelling (no contamination)	No visible change (no contamination)	Tube showed contamin
Placenta	No swelling (no contamination)	No swelling (no contamination)	No change observed (no contamination)

MS: Murashige and Skoog, NAA: α-naphthaleneacetic acid, BA: 6-benzylaminopurine

Results of Quantitative and Qualitative Estimation

Qualitative estimation result

TLC analysis using iodine fumes

The retention factor R_f values are listed in Table 5.

TLC analysis using Gibb's reagent

The plate however did not show the presence of any spots, and hence it did not prove as a useful method for the qualitative estimation of capsaicin.

TLC analysis for the callus extract

The callus extract was used as a sample and spotted on TLC plates. The callus extracts of control, on day 6 and day 9, were run for TLC analysis as shown in Table 6.

Quantitative Estimation

Results for capsaicin estimation by phosphomolybdic acid test

The equation $y = 0.54\ x + 0.038$ is used for further estimation of the sample concentration. The results are shown in Table 7.

The capsaicin estimation by phosphomolybdic acid test showed highest capsaicin content in callus extract after day 6 (29.829, 31.95) followed by day 9 (32.695, 30.523), day 3 (16.043, 14.844), *C. frutescens* (6.148, 5.7770, *C. annum* (5.962, 5.407), *Capsicum* (5.592, 5.222), and control (0.787, 1.443).

Results for total phenolic estimation by Folin's–Ciocalteu method

The equation $y = 0.6029\ x + 0.0119$ is used for further estimation of the sample concentration. The results are shown in Table 8.

The total phenolic estimation carried out by the Folin's–Ciocalteu method showed highest amount of phenols in callus extract after day 6 (11.098, 10.928) followed by day

Table 4: MS (basal)+2 mg/L 2,4-D+1 mg/L kinetin

Study parameter	Day 1	Day 7	Day 14	Day 24
Shoot	Slight swelling	Increase in swelling	Prominent initiation of callusing	Steady callusing, used for further tests
Node	Swelling	Slight initiation of callusing	Good callusing observed	Steady callusing, used for further tests
Internode	Swelling	Further swelling	Moderate callusing observed	Steady callusing used for further tests
Leaf	Prominent swelling	Initiation of callusing	Good callusing observed	Steady callusing, used for further tests
Flower	Very slight swelling	Increase in swelling in shoot region	Good callusing observed in the shoot area	Shoot region used for further tests
Placenta	No change	No change	No change	Tube discarded

MS: Murashige and Skoog, 2,4-D: 2,4-dichlorophenoxyacetic acid

Table 5: Results for TLC analysis – Iodine fumes

Plant material (fresh)	R_f value
Capsicum	Spot 1=0.185
	Spot 2=0.642
C. frutescens	Spot 1=0.785
C. annum	Spot 1=0.385
	Spot 2=0.685
	Spot 3=0.771

C. frutescens: Capsicum frutescens, C. annum: Capsicum annum, TLC: Thin layer chromatography

Table 6: TLC analysis of callus extract

Callus extract	R_f value
Control	Spot 1=0.692
	Spot 2=0.75
Callus (day 6)	Spot 1=0.714
Callus (day 9)	Spot 1=0.746

TLC: Thin layer chromatography

Table 7: Results for phosphomolybdic acid test

Study parameter	OD (660 nm) Set 1	Concentration mg/g	OD (660 nm) Set 2	Concentration mg/g
Capsicum	0.34	5.592	0.32	5.222
C. frutescens	0.37	6.148	0.35	5.777
C. annum	0.36	5.962	0.33	5.407
Callus extract (control)	0.05	0.787	0.04	1.443
Callus extract (day 3)	0.44	16.043	0.41	14.844
Callus extract (day 6)	0.51	29.829	0.53	31.095
Callus extract (day 9)	0.49	32.695	0.46	30.523

C. frutescens: Capsicum frutescens, C. annum: Capsicum annum, OD: Optical density

Table 8: Results for OD reading for Folin's–Ciocalteu test

Study parameter	OD (700 nm) Set 1	Concentration mg/g	OD (700 nm) Set 2	Concentration mg/g
Capsicum	0.19	2.954	0.21	3.285
C. frutescens	0.16	2.456	0.18	2.788
C. annum	0.12	1.793	0.11	1.627
Callus extract (control)	0.02	0.475	0.03	1.063
Callus extract (day 3)	0.09	2.790	0.11	2.506
Callus extract (day 6)	0.208	11.098	0.205	10.928
Callus extract (day 9)	0.125	7.324	0.127	7.457

C. frutescens: Capsicum frutescens, C. annum: Capsicum annum, OD: Optical density

9 (7.342, 7.457), *Capsicum* (2.954, 3.285), day 3 (2.790, 2.506), *C. frutescens* (2.456, 2.788), *C. annum* (1.792, 1.627), and control (0.475, 1.063).

CONCLUSION

Capsaicin has gained commercial importance due to its use in the medical, food, defense, pest, and sports industries. Due to its increased demand, the production on a large scale has to be facilitated. The only challenge being faced for the production of any secondary metabolite is the lack of knowledge about the biosynthetic pathway that exists in the plants which brings about the production of the metabolite. The same case being with capsaicin, its synthetic production does not match up to the demand levels.

The use of *in vitro* techniques for the production of secondary metabolites with the direct use of the callus is rare as compare to the use of immobilized cells. Cell cultures of Capsicum annuum L. were obtained from seedlings on MS medium supplemented with 2,4-D and kinetin. *In vitro*-grown cells and placental tissues from fruits were immobilized in calcium alginate. Ferulic acid fed to immobilized placenta at 2.5 mM level increased capsaicin production by 2-fold by the 5th day of the culture period. Production reached a level of 1345 μg capsaicin/g of immobilized placenta on the 14th day of culture. Production of capsaicin, on replenished nutrient medium in immobilized placenta was 2400 μg on the 30th day.

In the current project, the callus cultures of *C. frutescens* were grown and suspended in a liquid medium containing 2,4-D an kinetin in the presence of silver nanoparticles as an elicitor. The cells were not immobilized. The quantitative and statistical analysis shows significant increase in the amount of capsaicin as compared to the *Capsicum* fruit, even without immobilization. The highest amount of capsaicin content was found on the 6th day. The increase was approximately 2-fold in between the 3rd and the 6th day. This however brings down a conclusion that the nanoparticles acted as an elicitor and brought about capsaicin increase effectively.

In vitro methods for the production of secondary metabolites are being exploited to a wide range. There are significant challenges that are being faced for the synthetic production of the metabolites due to difficulty in decoding the biosynthetic pathway of the secondary metabolites. Hence, *in vitro* methods such as plant tissue culture and free cell suspensions in bioreactors prove as promising methods to obtain the product in its natural form. Extraction of the metabolite from these natural sources also proves easier and the product can be obtained in a much purified form.

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