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In vitro Effect of Various Growth Hormones in *Capsicum annuum* L. On the Callus Induction and Production of Capsaicin

A. Umamaheswari and V. Lalitha
Department of Biotechnology,
Prince Shri Venkateshwara Arts and Science College, India

Abstract: The present study was aimed to develop a novel protocol for the *in vitro* induction of callus for the production of capsaicin from *Capsicum annuum* L. For callus production young leaves, growing shoots, nodal region from the sterile germinated seedlings and placental regions and pericarp tissue from the fruit pods were used as explants. They were cultured on MS Medium supplemented with the various combinations of GA, IAA, NAA, 2, 4-D and Kin. Of all tried combinations of growth hormones, MS Medium with 2.0 mg L^{-1} 2, 4-D and 0.5 mg L^{-1} Kin was producing significant callus induction and proliferation in placental explants. The placental callus extract was taken for the estimation of capsaicin by colorimetric method. Extract had 1.6 mg^{-1} of capsaicin g^{-1} fresh weight of the callus. This could be an efficient protocol for capsaicin production from the placental calli and used for the large scale commercial production of capsaicin.

Key words: *Capsicum annuum* L., capsaicin, GA, IAA and Kin

INTRODUCTION

The genus *Capsicum* is a member of the Solanaceae family that includes tomato, potato, tobacco and petunia. The genus *Capsicum* consists of approximately 22 wild species and five domesticated species (Bosland, 1994). The five domesticated species are *C. annuum* L., *C. baccatum* L., *C. chinense*, *C. frutescens* L. and *C. pubescens*. *Capsicum annuum* L. is one of the major vegetable and spice crops grown world wide. It has become indispensable in every Indian home and also used medicinally, chutneys and pickles. Besides being an important food crops, Chilli peppers are used in pharmaceutical industries. Capsaicin, the main alkaloid responsible for pungency in chilies is used as a counter irritant balm form external application and it is also used in creamy to provide enhanced pain relief for arthritis patients.

The first attempt for the industrial production of secondary metabolites *in vitro* was made during 1950 to 1960 by the Pfizer Company and the first patent was obtained in 1956 by Routien and Nickell. Several kinds of bioreactors have been designed for large-scale cultivation of plant cells. In several cases cell cultures have been shown producing certain metabolites in quantities equal to (Kaul and Staba, 1967) or many fold greater than (Zenk, 1978) the parent plant. The level of pungency of the *Capsicum* species depends upon the concentration of capsaicinoids, primarily of capsaicin in the fruit. Capsaicin is the common name for 8- methyl N-vanillyl 6 nonamide, the chemical component most well known for its inhabitance in the internal white ribs of chili peppers and its ability to produce the burning hot sensation of chili-laced spicy foods. The present study was aimed at the production of capsaicin because of its reported medicinal effects such as carminative, digestive irritant, stomachic, stimulant, rubefacient and tonic (Simon *et al*., 1984). The plants have also been used as folk remedies for dropsy, colic, diarrhea, asthma, arthritis, muscle cramps and toothache. The *in vitro* production of

Corresponding Author: Dr. A. Umamaheswari Flat No.12, Plot No. 23, Ayya Arcade, Baby Nagar, Velachery, Chennai-600 042, Tamilnadu, India Tel: +91-44-22436565

secondary metabolites in plants can be regulated by the growth hormone used. Researchers in disciplines such as genetics and breeding, agriculture and technology have been interested in *Capsicum annuum* to develop new varieties with combinations of different optimal levels of the stimuli to maximize production of storable products for specific end uses. Physiologists have been intensely studying the action of the highly potent pungency stimuli. The compositional variations, biosynthesis of the functional components, the carotenoids, the volatile and the capsaicinoids are comprehensively reviewed (Govindarajan, 1986). Hence the present study focused on callus induction and production of capsaicin from *Capsicum annuum* through *in vitro* culture methods.

MATERIALS AND METHODS

The present research was carried out in the Biotechnology Laboratory at Prince Shri Venkateshwara Arts and Science College, Chennai (India) during August 2006 and January 2007.

Medium and Growth Hormones

The culture medium used for the present study was Murashige and Skoog (1962) Basal Medium. The medium was prepared by dissolving 4.27 g of MS Basal Medium which contains all the nutrients in 1 L of distilled water. In addition 0.44 g of calcium chloride, 3% sucrose and 0.8% agar were added to 1 L of the medium at pH 5.5.

The different growth hormones used were GA, IAA, NAA, 2,4-D, Kin. The stock solution was prepared as shown in Table 1. The growth hormones were filter-sterilized using Whatman filter Paper and a syringe filter.

Plant Material

Fertile seeds were collected from the pods of *Capsicum annuum* L. and were surface sterilized using 70% ethanol for 60 sec and sodium hypochlorite for 2 min followed by washes with sterile distilled water. The sterilized seeds were dried in a filter paper and inoculated in MS medium containing gibberillic acid at various concentrations. The explants were collected from the germinated sterile seedlings. Young leaves, growing shoots, nodal regions, were selected as explants. The placental regions and pericarp tissue from the pods were also used as explants.

Culture Initiation

The *in vitro* cultivation of plant tissues was done in a laminar air flow chamber. Absolute aseptic conditions were maintained to avoid microbial contamination.

Germination

The sterilized seeds were carefully taken and placed on the MS Medium slant in test tubes with GA. The inoculation was done inside the laminar air flow chamber. The tubes after inoculation were placed in light for 16 h provided by cool white light at 25±2°C.

Table 1: The prepared growth hormones concentrations

Growth hormone	Solvent used	Concentrations prepared (mg L^{-1})
GA	Distilled water	0.2, 0.4, 0.5, 1.0.
NAA	Ethanol	0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5.
IAA	Ethanol	0.1, 0.5, 1.0.
2,4-D	Ethanol	0.5, 1.0, 1.5, 2.0, 2.5.
Kin	HCl	0.1, 0.2, 0.3, 0.4, 0.5, 1.0, 1.5, 2.0.

GA: Gibberellic acid; NAA: a-naphtalene acetic acid; IAA: Indole-3-ethanoic acid; 2, 4-D: 2, 4-dichlorophenoxyethanoic acid; Kin: Kinetin 6-furfurylaminopurine

Leaf Explants Preparation

The sterile germinated seedlings were collected from the test tubes inside the laminar air flow chamber. The seedlings were cut with sterile blade for leaves and nodal regions of the stem. The explants were washed with 0.2% mercuric chloride for 2-3 min. The explants were then washed in sterile distilled water thrice to remove all the residual contents of mercuric chloride.

Inoculation of Leaf and Stem Explants

The leaves were taken and their edges near to midrib were trimmed and cut into bits of 1 cm. Each bit of leaf explant was then placed into the test tubes containing the culture media of different concentrations of growth hormones. The stem was cut into small pieces of about 2-3 cm in length and inoculated similarly.

Inoculation of Placental Regions

The pods of *Capsicum annuum* L. were surface sterilized and was cut longitudinally with the sterile blade to expose the placenta. The placenta was cut along the inner pericarp and made into small bits of 1 cm length. These were then placed into the test tubes with media using sterile forceps at 1-3 bits in a single tube.

Inoculation of Pericarp Tissue

The sterilized pods of *Capsicum annuum* L. were bisected longitudinally and the seeds and placenta were removed carefully after removing 4 mm portion at the two ends of the fruits. The pericarp tissue was cut into pieces (1×1 cm) and used for inoculation.

Callus Initiation

The explants were tried for callus induction in different concentrations and combinations of growth hormones.

Subculture

The responding cultures were sub cultured at 3 week culture period into fresh set of media and maintained under the same culture conditions.

Observation and Data Recording

The callus induction and proliferation were monitored at weekly intervals. The growth hormone combinations and the responses were also recorded.

Estimation of Capsaicin from Callus

Capsaicin was quantitatively estimated according to the method of Sadasivam and Manickam (1996).

RESULTS AND DISCUSSION

Plant cell culture offers a promising approach for large scale production of phytochemicals and has several advantages over whole plant production. Callus initiation involves three major considerations: selection of explant, medium and culture conditions (Hall *et al*., 1988). Capsaicin (8-methyl-N-vanillyl-6-noneamide), the principal pungent capsaicinoid, found only in the fruits of *Capsicum* species, is mainly synthesized in placenta and later transported to the other parts of the fruit (Iwai *et al*., 1979). Hypocotyl explants exhibits significantly better morphogenetic potential than other parts of the fruit (Pandeva and Simeonova, 1992), but their suspension cultures produce less capsaicin than fruits (Holden *et al*., 1987). Thus, in order to produce capsaicin commercially, it is desirable to get friable callus in sufficient amounts from high capsaicin synthesizing placenta tissue of highly pungent variety to obtain high capsaicin producing cell suspensions.

Table 2: Germination and callus induction from leaf, stem, pericarp and placental explants

Hormone combinations (mg L^{-1})					
GA	NAA	2,4D	Kin	IAA	Observation
0.2	-	-	-	-	No seed germination
0.4	-	-	-	-	Seed germination was observed in 5 days.
0.5	-	-	-	-	Seed germination was observed in 5 days
1.0	-	-	-	-	Seed germination was observed in 3 days and all the seeds were found to germinate
-	0.1	-	0.1	-	Stem - No response
-	0.2	-	-	0.1	Stem - No response
-	0.2	0.5	-	-	Callus growth at the edges of the stem
-	0.2	1.0	-	-	Callus growth at the edges of the stem
-	0.2	-	0.5	-	Callus growth at the edge of leaf
-	0.2	-	1.0	-	Wrinkling of leaf edges
-	-	0.5	0.5	-	Leaf - No response
-	-	1.0	0.5	-	Induction of callus of small size from leaf
-	-	1.5	0.5	-	Induction of callus of small size from leaf
-	-	2.0	0.5	-	Soft friable callus of large size was obtained from leaf
-	-	2.5	0.5	-	Stem - No response
-	0.3	-	0.5	-	Stem - No response
-	0.4	-	0.5	-	Leaf - No response
-	0.5	-	0.5	-	Leaf - No response
-	1.0	-	0.5	-	Small sized callus was seen in the leaf explant
-	1.5	-	-	-	Callus growth from nodal explant
-	-	-	0.1	0.5	Leaf - No response
-	-	-	0.2	0.5	Leaf - No response
-	-	-	0.3	0.5	Leaf - No response
-	-	-	0.4	0.5	Leaf - No response
-	-	-	0.5	0.5	Browning of the leaves.
-	-	1.0	0.5	-	The pericarp tissue turned brown and contamination was observed.
-	-	1.0	1.0	-	Callus induction in edges of the pericarp
-	-	1.5	0.5	-	Callus induction in edges of the pericarp
-	-	1.5	1.0	-	Pericarp - No response
-	-	2.0	0.5	-	Large sized callus growth was obtained in placental explant
-	-	2.0	1.0	-	Callus obtained from placenta was less proliferative
-	-	2.5	0.5	-	Callus growth was observed in pericarp
-	-	2.5	1.0	-	Pericarp - No response
-	-	0.5	-	0.5	Pericarp - No proliferation
-	-	1.0	-	0.5	Proliferation was seen in the stem callus
-	-	1.0	-	1.0	Placenta - No proliferation
-	-	2.0	0.5	-	Good proliferation of callus from placenta was observed
-	-	2.0	1.0	-	Proliferation of callus from leaf was seen
-	-	2.5	1.0	-	Less proliferation was seen from the leaf callus
-	-	2.5	2.0	-	Leaf - No proliferation
-	0.5	0.5	-	-	Callus formation throughout the stem
-	0.5	1.0	-	-	Proliferation of the stem callus
-	1.0	1.0	-	-	Callus proliferation on either edges of the stem
-	-	-	0.5	-	Proliferation of callus from leaf
-	-	-	1.0	-	Callus from leaf proliferated to a large sized soft callus
-	-	-	1.5	-	Callus from leaf proliferated to a large sized soft callus
-	-	-	2.0	-	Leaf - No proliferation was observed
-	-	1.5	0.5	-	Proliferation of callus from pericarp
-	-	1.5	1.0	-	Proliferation of callus from pericarp
-	-	1.5	2.0	-	Placenta - No proliferation

Germination of Seeds

Gibberellic Acid (GA) is a naturally occurring plant growth regulator which may cause a variety of effects including the stimulation of seed germination in some cases (Kabar, 1998). Germination of seeds was observed in MS Medium with 0.4 and 0.5 mg L^{-1} after 5 days of inoculation. But all the seeds inoculated were germinated in 1.0 mg L^{-1} GA concentration within 3 days (Table 2).

Callus Induction and Proliferation

Growth regulator concentrations in culture medium were critical for the control of growth and morphogenesis. Generally high concentrations of auxins and low concentration of cytokinins in the medium promote abundant cell proliferation with the formation of callus (Mohammad *et al*., 2003). Different explants were cultured *in vitro* to find out the most suitable explant for callus induction. Callus induction was observed in tubes with different concentrations of growth hormones (Table 2). MS Medium with 2 mg L^{-1}, 2-4 D and 0.5 mg L^{-1} kinetin was found to be optimal for callus induction and it gave a callus of 2.5 g fresh weight from single placental explants in about 30 days (Fig. 1). The result of the present work was similar to Rabindra *et al*. (2003). Mohammad *et al*. (2003) was found out that 2-4 D, IAA, BAP and kin were the most suitable growth hormones for the callus induction of wheat. Present study was very similar to Mohammad *et al*. (2003) except few growth hormone additions such as BAP and IAA.

Estimation Capsaicin

Capsaicin, an alkaloid, was used mainly as a pungent food additive in formulated foods. It was obtained from fruits of green pepper (*Capsicum* sp.). Capsaicin was also used in pharmaceutical preparations as a digestive stimulant and for rheumatic disorders (Sooch *et al*., 1977).

In callus cultures derived from pericarp and seedling explants of four varieties of *Capsicum annuum* viz., Punjab Surkh, Punjab Guccheder and Sweet Chilli that in Punjab Lal, the capsaicin content in seedling derived callus culture (7.28 mg g^{-1} dry weight) was comparable to that in fruits (7.00 mg g^{-1} dry weight). Capsaicin content in pericarp derived callus cultures of all the varieties was much higher than that in seedling-derived callus cultures and in fruits (Varindra *et al*., 2000).

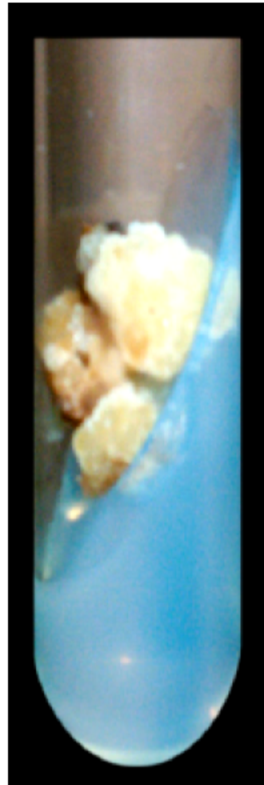

Fig. 1: Placental derived callus after 30 days of culturing

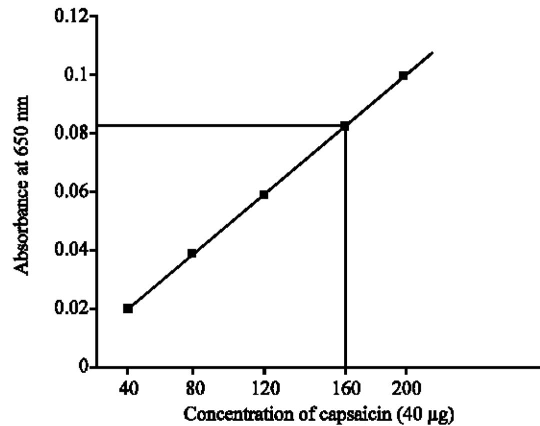

Fig. 2: Estimation of capsaicin from placental callus

Since the placental callus fresh weight was very high when compared to the stem explant, leaf explant and pericarp explant it was further taken for capsaicin estimation. A standard graph was plotted by using known concentration of capsaicin along the X-axis and absorbance along the Y-axis (Fig. 2). The amount of capsaicin present in the sample was calculated by using the standard graph. The amount of capsaicin in the placental extract was found to be 1.6 mg g^{-1} fresh weight of the callus. Sudha and Ravishankar (2003) found out that salicylic acid and methyl jasmonate individually enhance capsaicin production but when administered in combination there was no further enhancement in capsaicin production in cell suspension cultures of *Capsicum frutescence*. We have also worked out for the capsaicin production from callus of *Capsicum annuum* in a different approach.

Plant tissue culture is a noble approach to obtain their substances in large scale. Many companies in India and abroad are showing interest in this direction. Tissue culture is an alternative way for the production of phytochemical of therapeutic importance. The results obtained in present study could also serve as a potential alternate source for the large scale production of capsaicin for pharmaceutical purposes. Further, the vital enzymes in the biosynthetic pathways of capsaicin can be studied.

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