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In vitro pungency phenotyping in phenylalanine treated cell suspension cultures of seeds, pericarp and placenta of *Capsicum annuum* L. var. Yolo wonder and *Capsicum chinense* Jacq. var. Bhut jolokia.

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Abstract :
In vitro pungency phenotyping in phenylalanine treated cell suspension cultures the data revealed that 490.0 mAU/g of capsaicin was observed in phenylalanine treated placenta cell suspension cultures followed by 250.0 mAU/g in pericarp cell suspension cultures and 150.0 mAU/g in seeds cell suspension culture in Capsicum chinense Jacq. var. Bhut jolokia and while negligible capsaicin was observed in phenylalanine treated pericarp, placenta and seeds cell suspensions cultures of Capsicum annuum L. var. Yolo wonder

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I. Introduction:

Indian chilli peppers, have good export potential but need to conform to the pungency level prescribed by importing countries. There are instances of Indian chilli pepper consignment retuned back for non-maintenance of quality standards. Therefore, accurate measurement of pungency has become exigency (Carmichael, 1991). Moreover, food industry prescribes to reliable, safe and standard analytical methods for comparing pungency levels among different samples, before use in food and medicine.

Export market has motivated commercial farming of highly/extremely pungent chilli pepper. Capsaicin is one of the commonly consumed plant secondary metabolites (Govindarajan, 1987). Natural occurring genetic variation in capsaicinoids biosynthesis is critical to study biosynthetic pathway in order to identify pungency trait in chilli peppers. The knowledge of capsaicinoids biosynthetic pathway is important as both pungent and non-pungent peppers are valuable targets for vegetable crop improvement through plant breeding.

The objective of the present study was *in vitro* pungency phenotyping in phenylalanine treated cell suspension cultures of seeds, pericarp and placenta of *Capsicum annuum* L. var. Yolo wonder and *Capsicum chinense* Jacq.var. Bhut jolokia using HPLC.

II. Method:

Composition and Stocks of Murashige & Skoogs medium (MS, 1962) used in the present study.

Stock Solution:

Fifty milligrams (50 mg) of 2,4-dichlorophenoxy acetic acid (2, 4-D)was dissolved in 04 drops of 1.0 N NaOH and volume made up to 50 mL with distilled water (stock concentration - 1.0 mg/mL). The stock solution was stored in refrigerator until further use.

Preparation of Kn Stock Solution:

Fifty milligrams (50 mg) of Kinetin (Kn) was dissolved in 04 drops of 0.1N HCl and volume was made up to 50mL with distilled water (Stock Concentration-1.0 mg/mL). The stock was preserved in refrigerator until further use.

Preparation of MS Medium Stock Solutions:

Murashige and Skoogs medium stocknumbers I, II, III, IV, V and VI were prepared according to Table 1.

Table 1. Preparation of 1 liter MS medium using the stocks

MS Stock/Components	Volume (ml), Weight (mg) for 1.0 L
Stock I	10.0 ml
Stock II	1.0 ml

Stock III	10.0 ml
Stock IV	1.0 ml
Stock V	10.0 ml
Stock VI	1.0 ml
Glycine	200 mg
Myo-Inositol	100 mg
Sucrose	30000 mg
2,4-D (2.0 mg/L)	For callus induction and developing cell suspension cultures
Kn (1.0 mg/L)	For callus induction and developing cell suspension cultures

Preparation of MS Medium (Agar Solidified) for Callus Cultures:

One litre (1000 ml) MS basal medium (Table 1) supplemented with 2,4-D (2.0 mg/L) and Kn (1.0 mg/L) was prepared. The medium was solidified with 0.8% agar, by melting on water bath. The hot molten medium (15 ml) was distributed into culture tubes uniformly and the mouths covered tightly with cotton plugs wrapped with cheesecloth. The cultured tubes along with media were autoclaved at 121ºC /15 lbs pressure for 20 minutes, allowed to cool and kept in slanting position. This media was used to raise callus from seeds, pericarp and placenta of *Capsicum annuum* L. var. Yolo wonder and *Capsicum chinense* Jacq. var.Bhut jolokia.

Preparation of MS Medium (Agar-Free, Liquid Medium) for Cell Suspension Cultures:

One litre (1000 ml) MS basal medium (Agar-free, liquid medium) (Table 1) fortified with 2,4-D (1.0-5.0 mg/L) and Kn (1.0 to 2.0 mg/L) plus 100 µMof Phenylalanine was prepared. The pHof the medium was adjusted to 5.8 using 0.1 N NaOH. The liquid medium was dispensed into 100 ml conical flasks uniformly and mouths covered tightly with cotton plugs wrapped with cheesecloth. The conical flasks with liquid media were autoclaved at 121ºC /15 lbs pressure for 20 minutes, allowed to cool and the used in experiments. This media was used to raise cell suspension cultures from seed, pericarp and placental callus of *Capsicum annuum* L. var. Yolo wonder and *Capsicum chinense* Jacq. var.Bhut jolokia.

Surface Sterilization:

Fruits of *Capsicum annuum* L. var. Yolo wonder and *Capsicum chinense* Jacq. var.Bhut jolokia were washed with 1.0 % detergent (Tween-20 %) for 5 min, surface sterilized with mercuric chloride (0.1%) for 2 minutes and washed with distilled water. These fruits were treated with sodium hypochlorite (50% w/v) for 2 min and washed with sterile distilled water for 3 times and then placed on sterile filter paper to absorb the adhering water droplets. Seeds, pericarp and placenta were separated from the fruit and inoculated on MS medium.

Aseptic Techniques for Sterile Transfer:

All operations including, transfer and sterile filtration were made under laminar airflow cabinet (Klenzaids). Prior to operation, thelaminar airflow bench was swabbed with 70% alcohol to maintain maximum sterility. The scalpel, forceps and spatula were sterilized by flaming with alcohol before use inside the laminar airflow cabinet. The laminar airflow was switched on for 30 minutes before use to avoid the interruption for reversal of the airflow.

Inoculation:

Seeds, pericarp and placenta (explants) were inoculated separately in culture tubes containing MS (Agar solidified) + 2,4-D (2.0 mg/L) + Kn (1.0 mg/L) using forceps in a laminar air flow.

Seeds, pericarp and placental calli (100 mg each) were inoculated separately in conical flasks containing MS basal medium (Agar-free, liquid medium) + 2,4-D (2.0 mg/L) + Kn (1.0 mg/L) using a forceps in a laminar air flow.

Incubation:

Culture Room:

The culture tubes with seeds, pericarp and placenta (explants) were incubated at 25±2ºC under 16/8 hours photoperiod and the cultures were maintained at light intensity of 3000 Lux provided by cool white fluorescent lamps for 4 weeks.

Incubator Shaker:

The conical flasks with seeds, pericarp and placenta (explants) were incubated in incubator shaker at 100 RPM, Temperature of 25±2ºC and diffuse light for 4 weeks.

***In Vitro* Callus Induction**:

In vitro callus was induced in seed, pericarp and placenta were inoculated on MS + 2,4-D (2.0 mg/L) + Kn (1.0) mg/L in *Capsicum annuum* L. var. Yolo wonder and *Capsicum chinense* Jacq. var. Bhut jolokia, after four weeks of culture.

Fresh Weight and Dry Weight of Seed, Pericarp and PlacentaCalli:

The fresh and dry weight of seed, pericarp and placental calli was determined using a weighing balance. The mean value of fresh weight (mg) and dry weight (mg) of seeds, pericarp and placenta callus was scored using of at least 10 identical culture tubes.

Method:

- Twenty-five (25 ml) of cell suspension cultures of seeds, pericarp and placenta were centrifuged singly at 100 rpm and the pellet was air dried at 35 ^{0} C andmacerated using mortar and pestlewith acetonitrile solvent (1:10- gram: ml) and transferred to 120 ml screw cap bottles with Teflon lid.
- Screw cap bottles were incubated at 80 ^{0} C in a water bath for 4 h. The screw cap bottles were swirled manually every hour.
- The screw cap bottles were removed from the water bath and cooled to room temperature and centrifuged. The debris settled in the bottom and the supernatant on the top.
- The supernatant was filtered using Whatman No. 1 filter paper and stored in glass vials, capped and stored at 5^{0}C until further use.
- The supernatant of the seeds, pericarp and placenta was used singly for HPLC analysis.

Estimation of Capsaicin by HPLC (Bosland & Baral, 2007)

A 10-µl aliquot of the supernatant of seeds, pericarp and placenta were injected into injector port of Shimadzu- SPD 10A. The polar mobile phase -Methanol guided the sample into the column and the steady flow rate of pump was adjusted to 1.0ml/min. Retention time, height of peak and area of peak of capsaicin in seeds, pericarp and placenta was recorded.

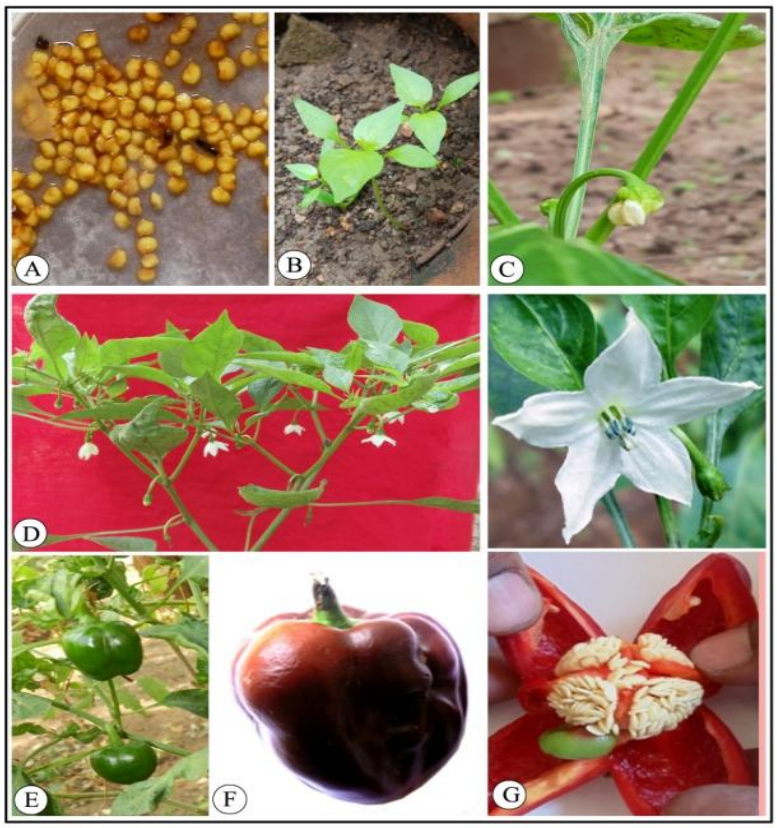

Fig:1 *Capsicum annuum* L. var. Yolo wonder :A. Yellow Colored Seeds,B. Young Seedlings , C. Two Months-Old Plant with Floral Bud , D. Three Months-Old Plant with White Flowers, E. Five Months -Old Plant with Bell Shaped Fruits F. Ripened Fruit, G. Internal Section of Fruit Showing Seeds attached to Placenta

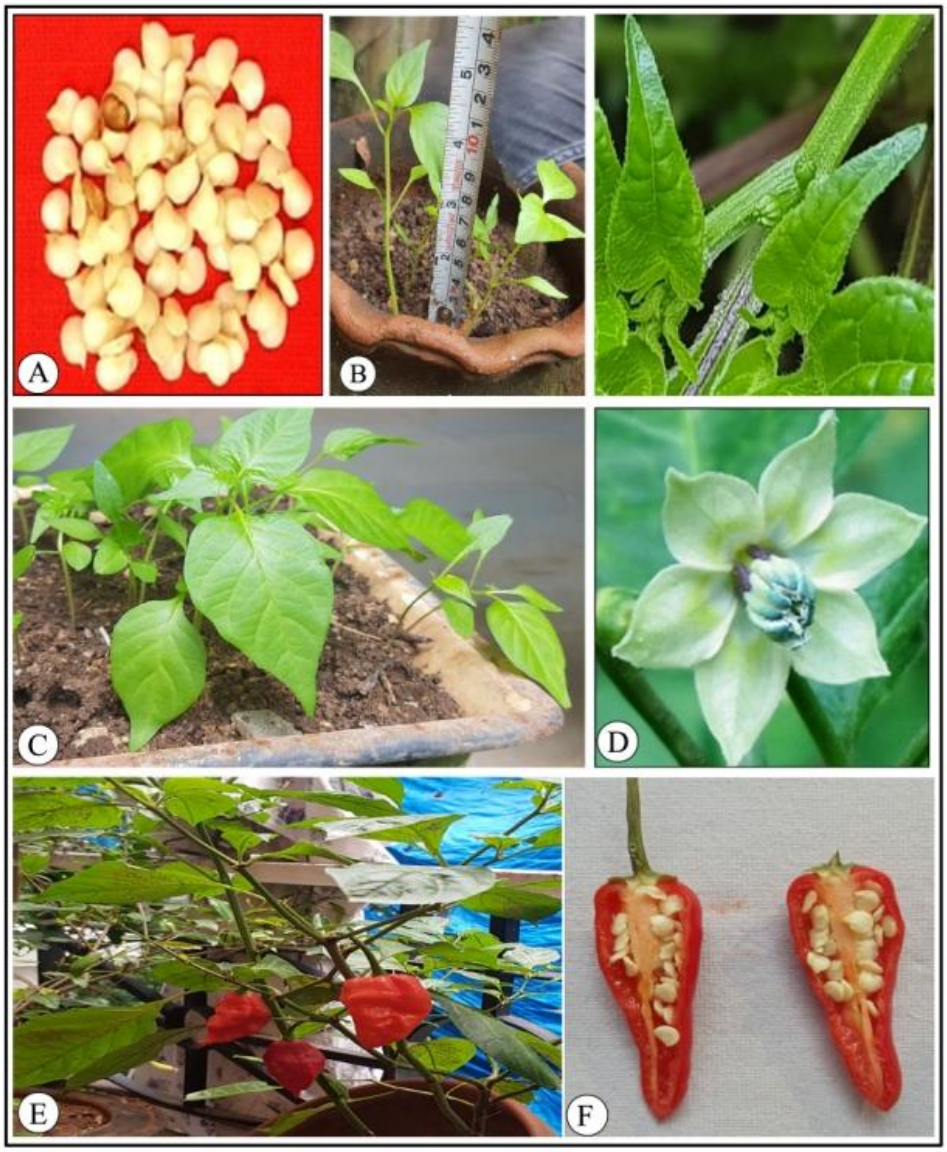

Fig:2 *Capsicum chinense* Jacq. var. Bhut jolokia, A. Yellow Colored Seeds, B. One Month-Old Seedlings , C. Two Months-Old Plant, D. Flower Showing Corolla with Green Tinge at the Throat, E. Five Months-Old Plant with Elongated Fruits with Undulating Surface , F. Internal Section of Fruit showing Seeds attached to Placenta

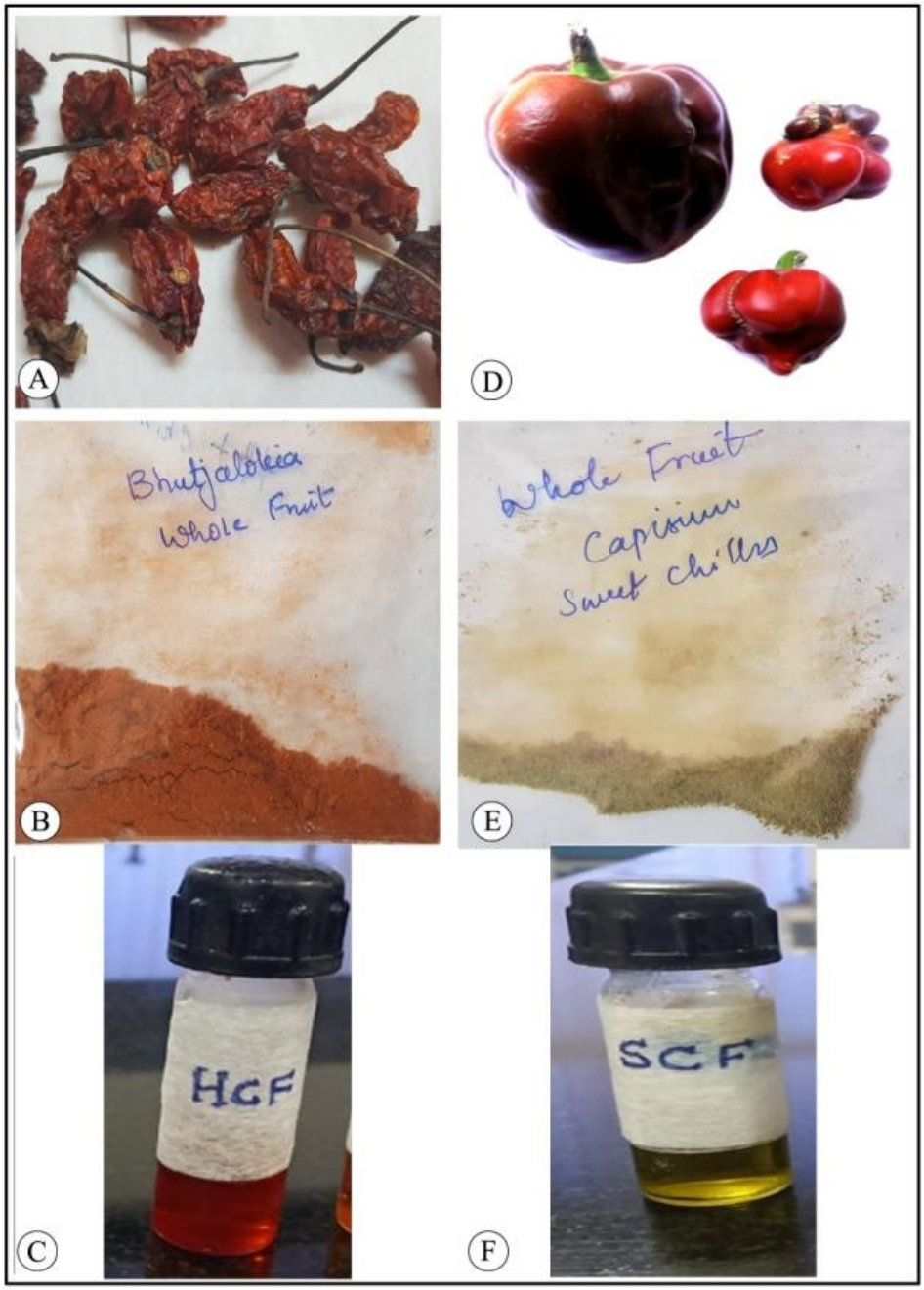

Fig:3 *In Vivo* Pungency Phenotyping: Preparation of Fruit (Seeds) Tissue for Capsaicin Estimation *Capsicum chinense* Jacq. Bhut Jolokia A. Fruits (Seeds) Tissue, B. Dry powder of Fruits (Seeds), C. Seeds Tissue extracted in Acetonitrile, *Capsicum annuum* L. Yolo wonder D. Fruits (Seeds) Tissue, E. Dry Powder of Fruits (Seeds), F. Seeds Tissue Extracted in Acetonitrile

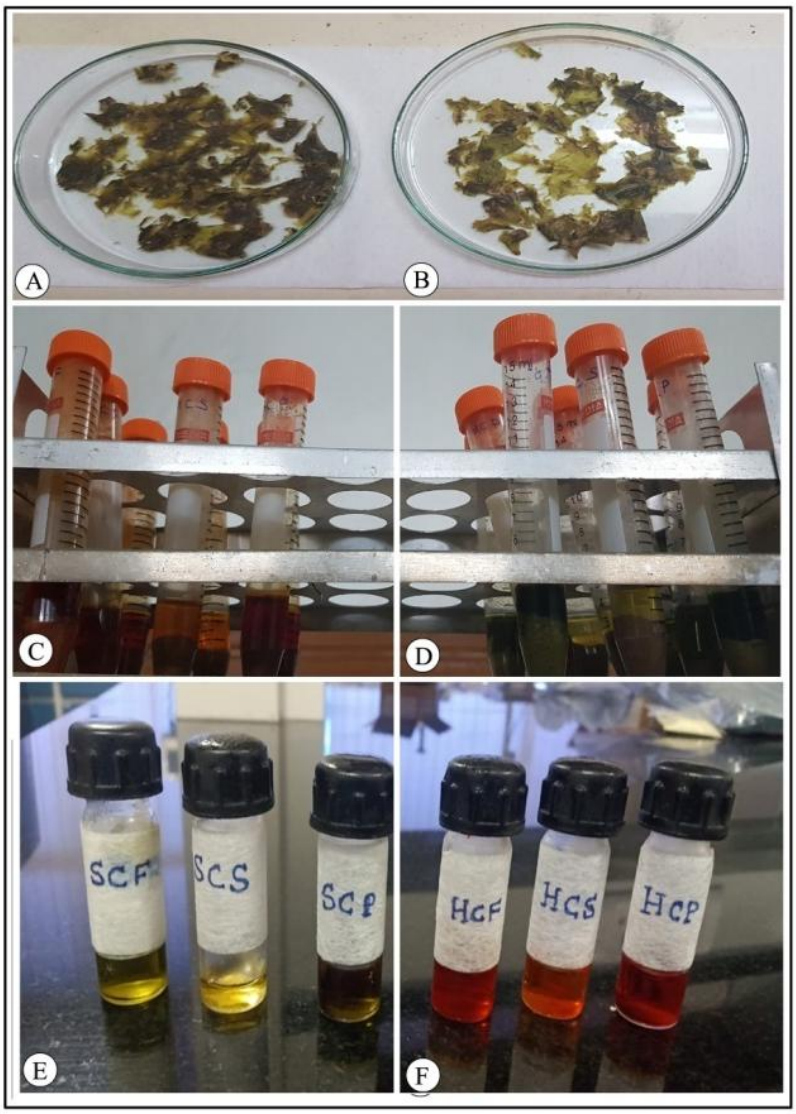

Fig:4 *In Vitro* Pungency Phenotyping: Preparation of Pericarp tissue for capsaicin estimation *Capsicum chinense* Jacq. var Bhut jolokia A. Pericarp Tissue C. Dry Powder of Pericarp, E. Pericarp Tissue Extracted in Acetonitrile. *Capsicum annuum* L. var. Yolo wonder, B. Pericarp Tissue, D. Dry Powder of Pericarp, F Pericarp Tissue Extracted in Acetonitrile.

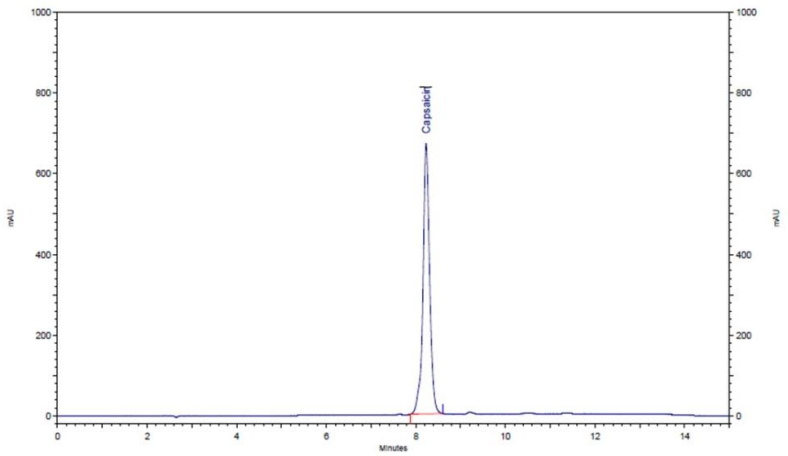

Fig:5 HPLC Chromatogram of standard capsaicin

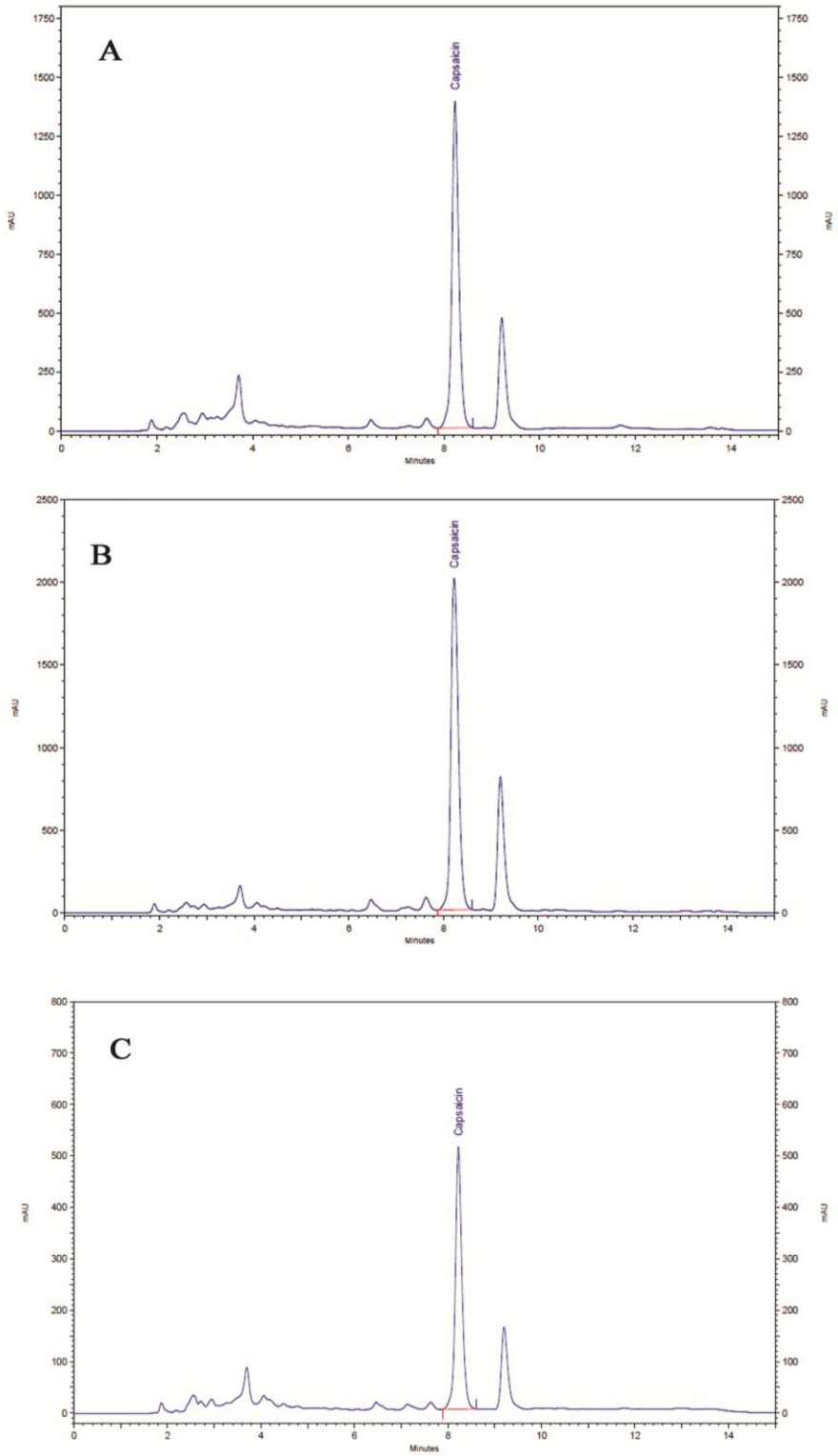

Fig:6 *In Vitro* Pungency Phenotyping: HPLC chromatogram of pericarp, placenta and seeds of *Capsicum chinense* Jacq. var. Bhut jolokia A. Capsaicin Chromatogram of pericarp tissue, B. Capsaicin Chromatogram of placenta tissue, C. Capsaicin Chromatogram of seed tissue

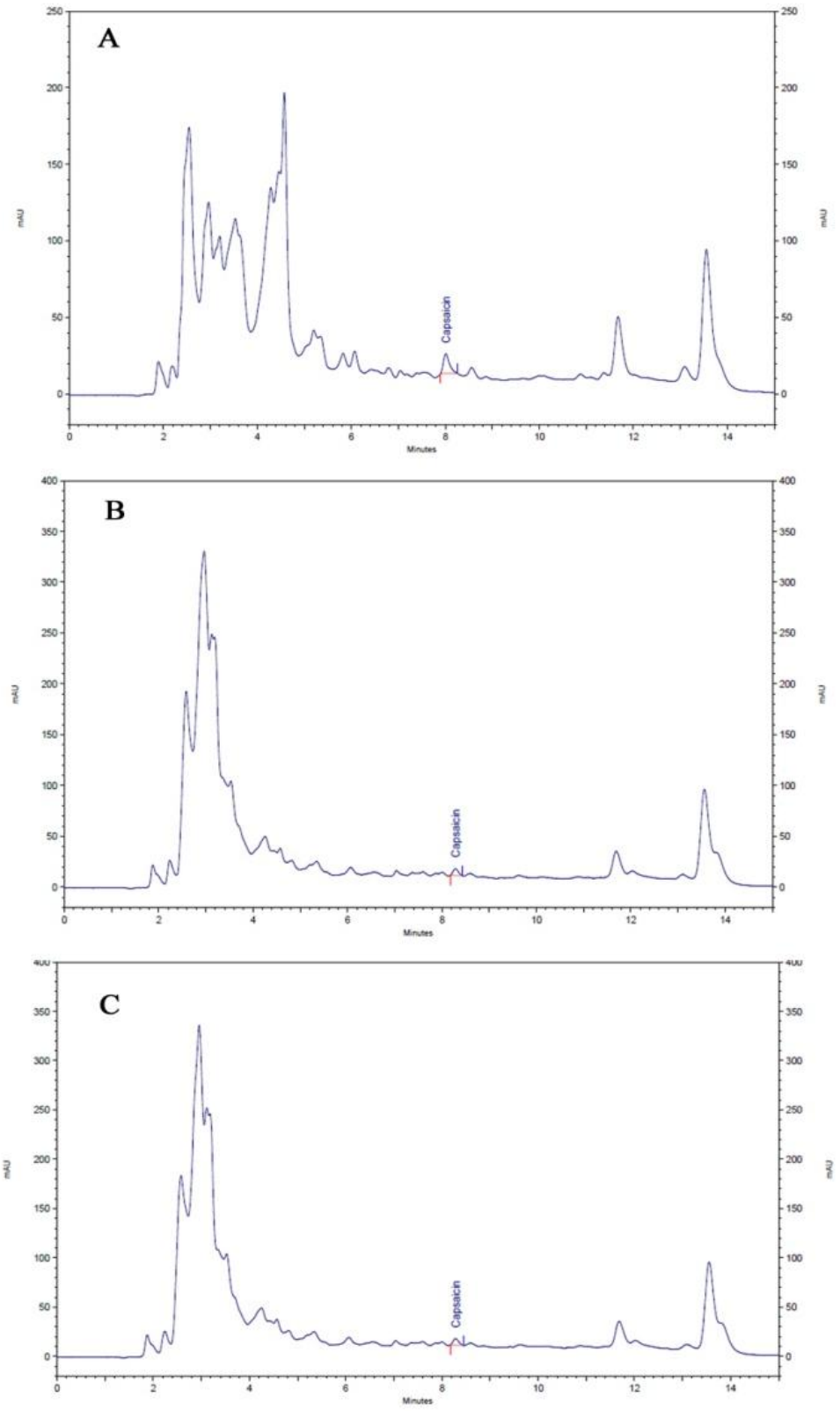

Fig: 7 *In Vitro* Pungency Phenotyping: HPLC chromatogram of placenta, seeds and pericarp of *Capsicum annuum* L. var. Yolo wonder A. Capsaicin Chromatogram of placenta tissue, B. Capsaicin Chromatogram of seeds tissue, C. Capsaicin Chromatogram of pericarp tissue

III. Results and Discussion:

Fresh and dry weights of seeds, pericarp and placenta calli in *Capsicum annuum* L. var.Yolo wonder and *Capsicum chinense* Jacq. var.Bhut jolokia was determined. The fresh weight of seeds, pericarp and placenta calli in both *Capsicum annuum* L.var.Yolo wonder and *C.chinense* Jacq. var. Bhut jolokia was about 100 mg. Friable callus developed in placenta while semi-compact to compact calli developed in seeds and pericarp.

Capsaicin was estimated in phenylalanine treated cell suspension cultures of seeds, pericarp and placenta of *Capsicum annuum* L. var. Yolo wonder and *Capsicum chinense* Jacq. var. Bhut jolokia respectively using HPLC. Phenylalanine treated cell suspension cultures of *Capsicum chinense* Jacq. var. Bhut jolokia produced small quantities of capsaicin.

The perusal of the data revealed that 490.0 mAU/g of capsaicin was observed in phenylalanine treated placenta cell suspension cultures followed by 250.0 mAU/g in pericarp cell suspension cultures and 150.0 mAU/g in seeds cell suspension culture *in Capsicum chinense* Jacq. var. Bhut jolokia and while negligible capsaicin was observed in phenylalanine treated pericarp, placenta and seeds cell suspensions cultures of *Capsicum annuum* L. var. Yolo wonder.

Earlier studies of organoleptic method and analytical methods was carried out in fruit tissue only, because capsaicinoids responsible for pungency accumulated in the placenta (Zamski *et.al.,* 1987; Suzuki *et.al.,* 1980). However, successful pungency phenotyping in non-fruit tissues like leaf cells Johnson *et.al.,* (1990) and leaf tissues (Rodriguez *et.al.,* 2012). Capsaicin production in cell suspension cultures of chilli pepper has been reported (Ravishankar *et.al.,* 1988; Lindsey & Yeoman, 1984). Biotransformation of externally fed protocatechuic aldehyde and caffeic acid to capsaicin in immobilized cell cultures and freely suspended cells of *Capsicum frutescens* has been reported (Rao & Ravishankar, 2000). Ochoa-Alejo, (2005) reported that addition of precursors and intermediates to in vitro cultures increase the production of capsaicin. Kehie *et.al.,* (2012) reported the osmotic effect of mannitol, sucrose and NaCl on capsaicin production in cell suspension cultures of *C. chinense.* Putrescine treatment influenced the capsaicin production in cell suspension cultures of *C. frutescens* (Govindaswamy *et.al.,* 2003). Salgado-Garciglia and Ochoa-Alejo (1990) reported increased capsaicin content in p-fluorophenylalanine-resistant cell cultures of *C. annuum.* The effects of nutritional stress on capsaicin production in immobilized cell cultures of *Capsicum annuum* were studied thoroughly by Ravishankar *et. al.,* (1988). Holden *et. al.,* (1988) have reported elicitation of capsaicin in cell cultures of *Capsicum. frutescens* by spores of *Gliccladium deliquescens.* Capsaicin was extracted from callus as well as from the medium (Johnson & Ravi shankar, 1990). Prasad *et.al.,* (2006) reported that by exogenous application of 8-methyl-nonenoic acid, it is possible to regulate capsaicin biosynthesis in *Capsicum* spp.

IV. Conclusion:

Capsaicin was observed in phenylalanine treated cell suspensions of seeds (150 mAU/g), pericarp (250 mAU/g) and placenta (490 mAU/g) of *Capsicum chinense* Jacq. var. Bhut jolokia but was absent in *Capsicum annuum* L. var. Yolo wonder. Hence, it is possible to augment capsaicin synthesis in phenylalanine treated cell suspension cultures of *Capsicum chinense* Jacq. var. Bhut jolokia

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