Chapter-3

Materials and Methods

3. Materials and methods

This investigation was carried out at the Experimental Farm of Indian Institute of Vegetable Research (IIVR), Varanasi. The plant material available with Chilli Breeding Unit of Crop Improvement Division were utilized during three consecutive seasons i.e. 2002-03, 2003-04, and 2004-05. The biochemical analyses were carried out in the Biochemistry Laboratory of Natural Resource Management Division of IIVR, Varanasi. In this chapter, plant materials utilized and methodology adopted are being described.

3.1 Plant materials

Season 2002-03

3.1.1 Collection of fruit samples

During the winter season (main growing season of chilli), red ripe fruits of a total of 127 germplasm lines including inbred lines, paprika lines (fruits with high colour and low pungency), landraces were collected in the month of February 2003 (Table 3.1). For most of the genotypes, fruits were collected from single plant. However, for morphologically non-uniform genotypes, fruits were collected from 3-5 plants and bulked before the analysis. In all the genotypes, dry matter content of the fruits was estimated. For the estimation of ascorbic acid contents, fresh red fruit samples of 105 genotypes were utilized. Powders from fresh red ripe fruits of 84 genotypes were utilized for spectrophotometric estimation of capsaicin estimation by spectrophotometer. Oleoresin was estimated in the fresh powder of 77 genotypes. Extractable

colour, colour value, and absorption ratio of fresh powder were estimated in 86 genotypes. To study the effect of storability on colour value in fourteen of these genotypes (LCA-235, Taiwan-1, 92-1203, IC-119310B, DC-3, DC-24, DC-6, DC-4, Bullet B-1, Bullet B-2, Bullet B-3, IR-8, BS-38, EC-391094), extractable colour, colour value and absorption ratio were also estimated from the six months stored powder.

Table 3.1. Chilli and sweet pepper genotypes utilized (2002-2003 and 2004-2005) for the estimation of biochemical parameters

SN	Genotypes	Parameters	SN	Genotypes	Parameters	SN	Genotypes	Parameters
1	LCA-357*	a, b, c, d, e	50	LCFKM	a, b, c, d, e	99	EC-345629#•	a, b, c,
2	PBC-535*	a, b, c, d, e	51	VNS-4	a, b, c, d, e	100	EC-345639	a, b, c, d, e
3	9852-173#	a, b, c, d, e	52	No 439	a, b	101	EC-341025	a, b
4	KA-2	a, b, c, d, e	53	No 476	a, b, c, d, e	102	EC-257216	a, b, d,
5	LCA-235#•	a, b, c, d, e	54	K.Chanchal	a, b	103	EC-341094#	a, b, c, e
6	Taiwan-1#•	a, b, c, d, e	55	BS-40	a, b, d, e	104	EC-391094	a, b, c, d, e
7	PBC-380*•	a, b, c, d, e	56	BS-20Y	a, b, c, e	105	EC-268216*	a, c, d, e
8	DC-16*	a, b, c, d, e	57	BS-21	a	106	EC-115657A	a, b,
9	9771-16#	a, b, c, d, e	58	BS-25	a	107	EC-119457B#	a, b, c, d, e
10	92-1206*	a, b, c, d, e	59	BS-22	a	108	EC-341075	a, b, d, e
11	9771-26up*•	a, b, c, d, e	60	BS-39Y	a, d, e	109	EC-257716	a, b, d
12	9852-18up#*	a, c, d, e	61	CCH-68	a, b, c, d, e	110	EC-491094	a, b
13	92-1203#•	a, b, c, d, e	62	MSH-96	a, b, c, e	111	JCA-283	a, b
14	977125-2	a, b, c, d, e	63	BS-19	a, c, d, e	112	Pusa Jwala	a, b, d, e
15	PBC-776*	a, c, d, e	64	CCH-100	a, b, c, d, e	113	JCA-9up	a, b, c, d, e
16	985218dn	a, c, d, e	65	ARH-1	a, b	114	Taiwan-2*	a, c, d, e
17	9771-26dn	a, c, d, e	66	Bullet-B2#	a, b, c, e	115	JCA-9dn	a, b
18	IC-119310B#•	a, b, c, d, e	67	Bullet-B1#•	a, b, c, e	116	JCA-352	a, b, e
19	IC-119368dn	a, b, c, d, e	68	IR-8#•	a, b, c, d, e	117	Pant C-1#	a, b, c
20	IC-119361*	a, b, c, d, e	69	Bullet-B3#	a, b, c, e	118	LCA-334*	a, b, c, d, e
21	IC-1402*	a, b, c, d, e	70	ISC-9	a, b	119	ARCH-50-1	a, b, c, e
22	IC-113368dn#	a, e	71	F-5-112	a, b	120	Phule Sai-30	a, b, c,
23	IC-119368up#	a, b, c, d, e	72	9852-190	a, b, c, d, e	121	IC-112474	a, b,
24	IC-119321*	a, b, c, d, e	73	PDAC-49A	a, b, c, e	122	JCA-601	a, b, c, e
25	IC-119457A	a, c, d, e	74	BS-34Y	a, b, c, d	123	BS-16	a
26.	IC-113367dn*	a, b, c, d, e	75	BS-38#•	a, b, c, d, e	124	BS-17	a
27	IC-119455#	a, b, d, e	76	BS-35*•	a, b, c, d, e	125	BS-5#•	a

SN	Genotypes	Parameters	SN	Genotypes	Parameters	SN	Genotypes	Parameters
28	IC-119474*•	a, b, c, d, e	77	BS-24	a, b, c, e	126	CH-1	a, b
29	DC-3#•	a, b, c, d, e	78	BS-29	a, b	127	Byad. Kaddi	a, b, c, d, e
30	DC-24#	a, b, c, d, e	79	BS-27	a, b, c, d, e	128	PBC-161#•	c, d
31	DC-7*•	a, b, c, d, e	80	Assam-10	a, b, e	129	DC-8#•	c, d
32	DC-6#•	a, b, d, e	81	BS-80	a, b, c, d	130	Per.-2A#•	c, d
33	DC-5*	a, b, c, d, e	82	BS-78	a, b, c, d, e	131	LCA-424#,	c, d
34	DC-4#•	a, b, c, d, e	83	BS-41Y	a	132	BS-13 R#•	c, d
35	DC-28	a, b, c, d, e	84	BS-79	a, b, c, e	133	CCA-4261#	c, d
36	LCA-206	a, b, c, d, e	85	BS-2	a	134	EC-519631#	c, d
37	PDAC-54B	a, b, c, d, e	86	BS-12	a, b	135	SM-20#	c, d
38	PDG-1*	a, b, c, d, e	87	BS-13#	a, b	136	J. Round#	c, d
39	NIC-268216#•	a, b, c, d, e	88	Assam-9	a, b	137	PBC-473#	c, d
40	LLS	a, b, c, d, e	89	Sandia	a ,c,	138	EC-119457#	c, d
41	PDG-1B	a, b	90	A. Local	a, b	139	Super#	c, d
42	Byad. Kaddi-A	a, b, c, d, e	91	JCA-283	a	140	IIHR-21#	c, d
43	Byad. Dabbi	a, b, c, d, e	92	Waialua	a, b, c,	141	IC-119367#	c, d
44	AKC 89/38*	a, b, c, d, e	93	No 537	a, b	142	PBC-142#	c, d
45	BC-30up	a, b, c, d	94	Perennial	a, b, c, d, e	143	EC-947635#	c, d
46	BC-4#•	a, b, c, d, e	95	Pant C-3	a, b	144	IC-109368#	c, d
47	BS-27	a, c, d, e	96	Phule Sai	a, b	145	Arka Abhir#•	c, d
48	Kaala	a, b, c, d, e	97	Punjab Lal	a, d, e			
49	Japani Longi	a, b	98	NuMex P.#	a, b, c, d, e			

Note:

a-e : Estimated biochemical parameters; a - dry matter; b - ascorbic acid; c - capsaicin; d - oleoresin, e- extractable colour, colour value and absorption ratio.

* : These genotypes were analyzed for capsaicin, oleoresin, extractable colour, colour value and absorption ratio during two growing seasons (2002-03 and 2004-05).

\# These genotypes were analyzed for capsaicin and oleoresin during 2004-2005.

• : These genotypes were analyzed for capsaicin following two-methodologies (spectrophotometer and HPLC).

3.1.2 Crosses developed

Season 2002-03

A total of 63 cross combinations were developed utilizing two cytoplasmic-nuclear male sterile CMS lines, *viz*. CCA-4261 and CCA-4757 as female parent and 62 genotypes (mostly hot pepper) maintained through at

least two generations of selfing, as a male parent in crossing programme. The progenies of these 63 CMS based crosses (F_1s) were evaluated during next season (2003-04) in order to examine fertility restoration and suggested presence of restorer (*Rf*) or maintainer (*rf*) allele in the male inbred lines (Table 3.2).

Season 2003-04

Five CMS lines, *viz*. CCA-4261, CCA-4757, MS-1, MS-2 and MS-4 were utilized as a female parent and 50 inbred lines bearing pungent, mildly pungent and non-pungent fruits were utilized as a male parent in crossing programme (Table 3.3). A total of 67 cross combinations were developed and during the next season (2004-05), these F_1s were evaluated for fertility restoration. Red ripe fruits of 10 crosses and their parents were utilized for the analysis of capsaicin, oleoresin, extractable colour, colour value and absorption ratio. Besides fertility testing of 10 F_1s, the observations were recorded for fruit length, fruit width, fruits per plant, ten fruits weight and plant height.

Season 2004-05

3.1.3 Collection of fruit samples

During this season, red ripe fruits of 67 genotypes were collected along with the red ripe fruits of 30 Recombinant Inbred Lines (RILs) (Table 3.4) for the analysis of capsaicin, oleoresin, extractable colour, colour value and absorption ratio. These RILs (F_8 generation) have been derived from a cross between sweet pepper (California wonder) and hot pepper (LCA-235) at IIVR Varanasi (Kumar *et al.,* 2000b). Among the 67 genotypes, 21 genotypes were the same that were collected during 2002-2003 (* genotypes in Table 3.1) and 46 genotypes were those, which were analyzed for capsaicin and oleoresin (# genotypes in Table 3.1). In 24 genotypes (• genotypes in Table 3.1)

capsaicin was estimated following two-methodologies, viz; spectrophotometer and HPLC. In 30 RILs capsaicin was estimated by HPLC.

Table 3.2. List of parental lines used to develop crosses during 2002-2003

CMS line	Male parents
CCA-4261	PBC-380, ISPN-2-3*, PBC-473*, SP-10, Haricharan, SP-23B, California Wonder*, BS-85, SP-43B, KTCPH-5, AKC-89/38, SP-33B, SP-93B, Indam-5*, BRC-1, Super, Waialua, KDCS-810, DC-2, DC-6, DC-28, F5-112, Kaala*, BS-27, ISPN-2-1*, SP-106, SPG-103, SP-177, SP-79B, PBC-212, DC-7, DC-24, PBC-367, DC-3, DC-8, DC-24, BS-13, BS-35, BS-2, BS-40, BS-20, BS-38, BS-5, PBC-210, BS-79, BS-28, BS-4, Pant C-1, P-15, BS-34, MS-12, DC-4, No-143, Hot line, BS-85, Indam*, PRC-1
CCA-4757	9852-173, KA-2, LCA-235, Punjab Lal, PBC-367, PBC-535

* Sweet pepper genotypes

Table 3.3. List of parental lines used to develop crosses during 2003-2004

CMS lines	Male parents
MS-1	KA-2, PBC-380, PBC-473, PBC-534, Kaala*, Punjab Lal, SP-55C, SP-23B, SP-106, Pusa Jwala, KSPS-501*
MS-2	PBC-380, KA-2
MS-4	Punjab Lal, PBC-534, KA-2, PBC-473, SP-106, Pusa Jwala
CCA-4261	Kaala*, LCA-357, SP-31-C, P-1649, California Wonder*, KSPS-501*, KDCS-810, SM-20, Picadore*, EC-519605, MI-2, K. Chanchal, Pusa Deepti*, PBC-535, KSPS-202*, Phule Sai, EC-519631, F5-112, NIC-268216, Perennial, Pant C-1, SP-106, Taiwan-2, PBC-473, BS-13, Pusa Jwala, BS-40, BS-85, Punjab Lal, SP-4C, EC-519635, Greygo, ISPN-2-3*, SPG-103, Japani Longi, BS-27, KA-2, EC-519593*, EC-519607*, EC-519612*, Byadagi Kaddi, EC-519594*, EC-519694*, EC-519589*, EC-519585*, Byadagi Dabbi
CCA-4757	Pusa Jwala, PBC-473

* Sweet pepper genotypes

In three of 67 genotypes (Taiwan-1, CCA-4261, Pant-C-1) capsaicin was estimated in fresh as well as in twelve months stored powder. Ascorbic acid (vitamin C) was analyzed in green and red ripe fruit of 11 promising

genotypes, *viz*. KA-2, PBC-535, 9852-18, BS-38, Taiwan-1, 9852-173, BS-35, 92-1206, IC-119474, IC-119321, 97-7126.

Table 3.4. List of RILs were utilized for biochemical analysis

C3F7 PT-26D	C3F7 PT-14B	C3F7 PT-20E	C3F7 PT-20B	C3F7 PT-5
C3F7 PT-26	C3F7 PT-22E	C3F7 PT-3	C3F7 PT-26A	C3F7 PT-26E
C3F7 PT-18B	C3F7 PT-6B	C3F7 PT-22A	C3F7 PT-13	C3F7 PT-33A
C3F7 PT-6B1	C3F7PT12(OP)	C3F7PT-6(OP)	C3F7 PT-33C	C3F7 PT-4
C3F7PT-39A4	C3F7 PT-2	C3F7 PT-22B	C3F7 PT-13B	C3F7 PT-11
C3F7 PT-14D	C3F7 PT-7	C3F7 PT-18A	C3F7 PT-10	C3F7 PT-39A

3.2 Methodology

3.2.1 Field experiments

3.2.1.1 Raising of crops

Seeds of all the plant materials described above were shown in nursery beds during the month of July. Proper seedling management practices were exercised in order to raise healthy seedlings. In general 30 days old seedlings were transplanted on raised bed at a distance of 60 x 45 cm.

3.2.1.2 Development of crosses

Crosses were developed utilizing cms lines i.e. CCA-4261, CCA-4757, MS-1, MS-2 and MS-4 as female parent, whereas, germplasm and inbred lines as male parents. Pollens were collected by opening the closed mature bud of desired male parent. The mature bud of CMS line was opened very carefully using pointed forcep. Manual pollination was carried out without causing any damage to the stigma. The pollinated buds were covered with cotton and labeled using small tags. The mature red ripe fruits were collected, dried and seeds were removed, stored in a well labeled envelop for sowing in the next season.

3.2.1.3 Determination of fertility restoration

Five randomly selected plants of each F_1 cross were evaluated for fertility restoration. The ability of F_1 plants to set selfed fruits with seeds is usually considered as fertility restoration in F_1 (male fertile F_1) and non-ability of the F_1 plants to set a selfed seed is considered as non-restoration in F_1 (male sterile F_1). However, during this study, male fertile crosses (involvement of restorer male parent) and male sterile crosses were determined using following two methods:

i. Anthers of fully opened flowers were tapped on the nail and examined the presence (male fertile) or absence/very few (male sterile) of pollen grains on nail.

ii. Bagging of closed buds (before fertilization) with the help of muslin cloth bag and examining ability (male fertile) and non-ability (male sterile) of plants to produce selfed seeds.

3.2.1.4 Recording the morphological observations

Five randomly selected plants of F_1s and parents were utilized to score observations on selected morphological characters (IPGRI, 1995).

Fruit length (cm): Ten fruits were harvested randomly from each cross and parent. Length was measured and expressed as average.

Fruit width (cm): Fruit width was also measured on the randomly harvested 10 fruits from each parent and cross.

Fruits/plant: Total numbers of fruits harvested from five randomly selected plants and were counted.

Fruits weight: Ten randomly selected green fruits were weighted and expressed as 10 fruit weight.

Plant height (cm): Height of five randomly selected plants was measured at physiological maturity stage.

3.2.1.5 Heterosis estimation

Ten cross combinations were selected for heterosis analysis. Better parent heterosis (heterobiltrosis) and mid parent heterosis of five biochemical parameters and five morphological characters, *viz.* capsaicin, oleoresin, extractable colour, colour value, absorption ratio, fruit length, fruit width, fruits per plant ,10 fruit weight, plant height were calculated utilizing following formula:

$$\text{Heterosis \% (BP)} = \frac{F_1 - BP}{BP} \times 100$$

$$\text{Heterosis \% (MP)} = \frac{F_1 - MP}{MP} \times 100$$

F_1 - Mean value of F_1 cross; BP - Mean value of better parent; MP- Average of both the parent

3.2.2 Biochemical analysis

3.2.2.1 Determination of dry matter

For the analysis of dry matter, fruits at red ripe stage were harvested and kept in well-labeled envelopes. Fresh weight of each fruit sample was taken on digital analytical balance. Thereafter samples were shade dried for 2-3 days and then kept in an hot air oven at 60±2°C till they attained constant weight. The complete dried samples were then taken out from the oven and weight of each sample was again recorded immediately. The dry matter percent was calculated as follows:

$$\text{Dry matter \%} = \frac{\text{Dried weight of the sample}}{\text{Fresh weight of the sample}} \times 100$$

3.2.2.2 Estimation of ascorbic acid

Ascorbic acid was estimated titrimetrically using 2, 6-dichlorophenolindophenol method (Sadasivam and Thymoli, 1987). Five ml of the working standard solution was pipetted out in a conical flask then 10 ml of 4.0% oxalic acid was added and it was titrated against the dye till the appearance of pink colour. The amount of dye consumed was equivalent to the amount of ascorbic acid. After standardization known weight of the fresh fruit sample was grinded in 4.0% of oxalic acid with the help of pestle and mortar and final volume made up to 50 ml. This was filtered and 10 ml of filtrate were pipetted out in a conical flask to which 10 ml of oxalic acid (4.0%) was added and solution was titrated against the dye. Burette reading was recorded at the point at which colour of the solution changed to pink.

Ascorbic acid content was calculated by the following formula:

$$\text{Ascorbic acid (mg/100g)} = \frac{0.50\text{ mg}}{V1} \times \frac{V2}{10\text{ ml}} \times \frac{50\text{ ml}}{\text{Weight of sample}} \times 100$$

[Preparation of working standard: 100 mg ascorbic acid was dissolved in 100 ml (final volume) of oxalic acid. Then 10 ml of this solution was pipetted out and further dissolved in 100 ml (final volume) of oxalic acid]

3.2.2.3 Capsaicin estimation

3.2.2.3.1 Capsaicin estimation by spectrophotometer

Capsaicin content in chilli powder was estimated by the method of Thimmaiah (1999). Harvested red ripe fruits were dried in an oven at 60 ± 2^0.C until it was completely dehydrated. Samples were ground to fine powder and passed through a 2 mm sieve. For the extraction of capsaicin, 500 mg of powder was taken in a centrifuge tube and dissolved in 10 ml of dry acetone

by continuous shaking on a mechanical shaker at room temperature for 3-5 hours. Thereafter, samples were centrifuged for 10 minutes at 10,000 rpm, and after centrifugation, 1 ml of supernatant was pipetted out in a test tube. The supernatant was evaporated to dryness on a hot water bath. The residue was dissolved in 5 ml (0.4%) NaOH and 3 ml (3.0%) phosphormolybdic acid by vigorous shaking. The solution was gently shaked using vortex and incubated for one-hour and solution was filtered into centrifuge tubes and centrifuged for 10 min at 5000 rpm. The absorbance of the sample was recorded at 650 nm using UV-visible double beam (Shimadzu UV-1601) spectrophotometer. The blank solution contained 5 ml 0.4% NaOH and 3 ml (3.0%) phosphomolybdic acid. The capsaicin percentage was calculated by following formula:

$$\text{Capsaicin (\%)} = \frac{\mu g \text{ Capsaicin}}{1000 \times 1000} \times \frac{100}{1} \times \frac{100}{2}$$

Standard calibration

Preparation of stock standard: 10 mg of standard capsaicin (Sigma, US) was dissolved in 10 ml of 0.4% NaOH (1000 ppm) solution and used as stock solution for standard calibration.

Preparation of working standard: Working solution of 200, 160, 120, 80, 40 ppm were prepared by diluting the stock standard (1000 ppm) by adding 0.4 % NaOH solution. The absorbance (650 nm) of working solution 40 ppm, 80 ppm, 120 ppm, 160 ppm and 200 ppm were recorded and by using this a standard curve was plotted.

3.2.2.3.2 Capsaicin estimation by HPLC

The capsaicin content was also estimated using HPLC following the method of Collins et al (1995). Samples and standard were subjected to HPLC

analysis by isocratic elusion. Mobile phase comprised of a mixture of methanol and water (30:70 v/v) at a flow rate of 1ml/min. The HPLC system comprised of C_{18} reverse phase column (5 µm; 150 x 4.6 mm i.d.), LC-10 AT (Shimadzu) quaternary gradient pump, SCL-10 A (Shimadzu) controller controlled with a millennium data acquisition software, SPD-10 AV uv-visible detector, DGU-14A degasser and CTO-10AS column oven. Detection was done at 280 nm wavelength. Capsaicin was identified and quantified by comparing the peak area at retention time of 4.87 minute as recorded for standard peak with known concentrations using the following equation:

$$\text{Capsaicin ((µg/g)} = \frac{\text{Area of sample}}{\text{Area of standard}} \times \frac{\text{Concentration of standard}}{\text{Weight of sample}} \times \text{Dilution factor}$$

Hundred mg powder of each sample was dissolved in 10 ml of acetonitrile by heating at 80^0 C for 4 hours. The samples were allowed to cool at room temperature until the supernatant particles settled down. The supernatant was filtered using 0.45 µ Millipore filter with the help of a syringe. The filtrates were collected in a 1 ml capacity vial and stored at 0-4°C till further analysis. 20 µl aliquot was utilized for each HPLC injection.

Standard calibration: Standard of 8 methyl-n-vanillyl-6-nonenamide (capsaicin) obtained from Sigma Chemical Co; St. Louis, MO, USA was used for calibration. Standard solutions of 500, 250, 100 and 50 ppm were prepared in methanol by dilution of a 1000-ppm stock solution.

3.2.2.4 Oleoresin estimation

Oleoresin percentage was estimated as suggested by Mathew et al., (1971). Capsicum oleoresin was extracted from the powder of dried chilli fruits by continuous shaking in acetone for 4-6 hrs on a mechanical shaker at room temperature and after that the extract was allowed to reflux on a clevenger

apparatus for 4-6 hrs. The viscous liquid derived after percolation was further evaporated to near dryness on a buchi type rotatory evaporator and the oleoresin percent was calculated as follows:

$$\text{Oleoresin \%} = \frac{\text{Dried weight of the residue}}{\text{Fresh weight of the sample}} \times 100$$

3.2.2.5 Estimation of extractable colour, colour value and absorption ratio

The procedure as described in AOAC (1996) was used to determine the extractable color, color value and absorption ratio. Acetone (5 ml) was used to dissolve 20 mg of grinded powder by continuous shaking. This process was repeated by adding 5 ml of acetone in the sample followed by continuous shaking. The absorbance was recorded at 455 nm, 460 nm, 462 nm and 470 nm using a UV-visible double beam (Shimadzu uv-1601) spectrophotometer. The absorbance was adjusted in the range of 0.25-0.50. Blank reference was set using acetone. Then the extractable colour, colour value and absorption ratio were calculated using following formulas:

$$\text{Extractable colour} = \frac{\text{Absorbance at 460 nm} \times 16.4 \times I_f}{\text{Sample weight (g)}}$$

I_f = Instrument correlation factor

$$I_f = \frac{\text{Declared absorbance of Glass Reference}}{\text{Absorbance obtained at 465 nm on glass reference standard}}$$

$$\text{Colour value} = \frac{\text{Absorbance at 462 nm} \times 6600}{\text{Sample weight (g)}}$$

$$\text{Absorption ratio} = \frac{\text{Absorbance at 470 nm}}{\text{Absorbance at 455 nm}}$$

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