CHAPTER III

MATERIALS AND METHODS

1. Varietal collection and inbred seeds production

Local bitter gourd accessions were collected from many provinces of Thailand. Each accession was self-pollinated and was selected for similarity to the mother plant for inbred seed production. One accession was chosen from commercial seed for a standard variety.

2. Yield and horticultural characteristics

Inbred seeds of 13 local bitter gourd accessions were planted in February 2000. Randomized complete block design (RCBD) with 4 replications was used. Plot size of each accession was 4 × 6 m^2. There were four rows in each plot and six plants in each row. Spacing was 1 m between rows and 1 m between plants. Chemical fertilizer of 15-15-15 was applied at the rate of 8 g/plant (12.8 kg/rai) before transplanting, and it was applied at the same rate at 21 days and 42 days. Staking was done when the plants had 3 or 4 true leaves. Subsequently, vines were tied as necessary. Irrigation was done after transplanting and repeated every 2-4 days. Insecticides such as carbaryl and carbosulfan were sprayed to control pest. Fresh seeds from self-pollinated ripe fruits from border rows of each accessions would be used for 30 kilodalton (30 kDa) protein extraction. Harvesting was started on March 9, 2000 and finished on April 24, 2000. Data were collected as follows: -

2.1 Botanical characteristics

2.1.1 Leaf; mature leaves of each accession were measured:

a. leaf size (width, length) and petiole lengths.

b. apex shape, recorded the apex shape by using plant identification terminology (Harris and Harris, 1994 and Jones and Luchsinger, 1987).

c. color of mature leaves of each accession was measured by using Color QUEST II Hunter Lab (DeMan, 1999). The Hunter L, a, b system can be represented by the color space which shown in Figure 1. The L, a and b values could be converted to CIE values in the CIE chromaticity diagram which shown in Figure 2 (DeMan, 1999) by the equations: -

$$x = \frac{a + 1.75L}{5.645L + a - 3.012b}$$

$$y = \frac{1.786L}{5.645L + a - 3.012b}$$

2.1.2 Flowers

a. width and length of petals and length of peduncle of pistillate and staminate flowers were measured.

b. width, length and distance to peduncle base of sessile bracts of pistillate and staminate flowers of each accession were measured.

2.1.3 Fruit; fresh fruits at the same pollination age (days) of each accession were measured:

a. shape of fresh fruit, by using plant identification terminology (Harris and Harris, 1994 and Jones and Luchsinger, 1987).

b. skin color, skin color of fresh fruits were measured by using Color QUEST II Hunter Lab. The Hunter L, a, b system could be represented by the color space which shown in Figure 1. The L, a and b values could be converted to CIE values in the CIE chromaticity diagram which shown in Figure 2.

c. fruit peduncle length.

d. fruit base to sessile bract.

e. fruit size (length and diameter).

f. fruit length/diameter (L/D) ratio.

g. flesh thickness, measured the flesh thickness in millimeter by using verneer caliper.

2.1.4 Seed; dry seed size was measured:

a. number of seeds per ripe fruit.

b. width, length and thickness were measured by using verneer caliper.

c. correlations of seed size and number of seeds/ripe fruit were analyzed.

2.2 Fresh fruit were harvested from double rows in the middle of each plot excepted the first and final plants. Data were collected as follows: -

2.2.1 weight of fresh fruit.

2.2.2 number of fruits per plant.

2.3 Other horticultural characteristics (Jizhe, 1993) as follows:

2.3.1 number of days after seed germination (the days after seed germination were started measuring at 75% seed germination) to 50% female flower.

2.3.2 number of nodes of first female flower.

2.3.3 number of female and male flowers on the main stem and lateral branches of the first 25 nodes for 21 days (February 22-March 13, 2000).

2.3.4 female to male flower ratio on the main stem and lateral branches of the first 25 nodes for 21 days (February 22-March 13, 2000).

2.3.5 days from anthesis of fresh fruit.

2.3.6 number of days after seed germination to first harvest.

2.3.7 number of days after seed germination to last harvest.

2.3.8 number of harvesting times.

2.3.9 main stem lengths at last harvest.

2.3.10 number of lateral vines at last harvest.

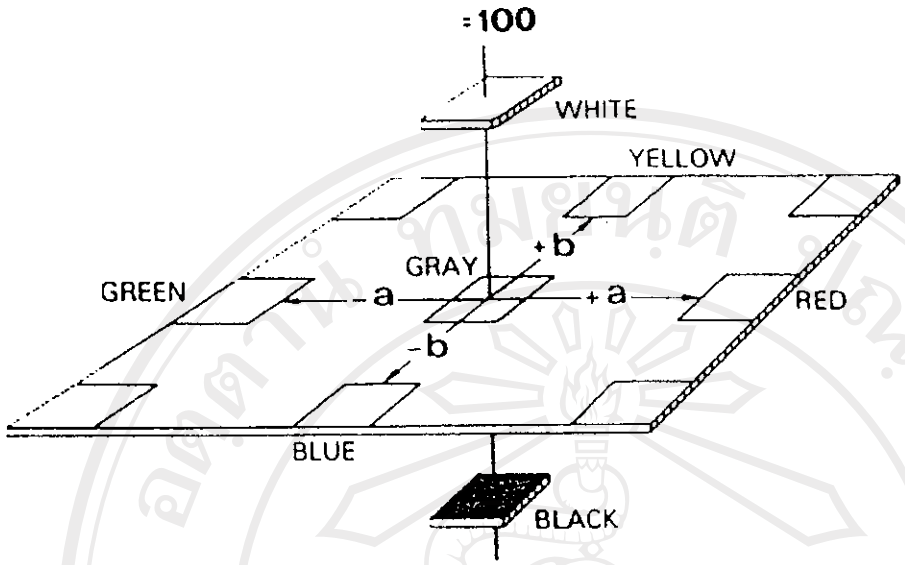

Figure 1 The Hunter L, a, b Color Space (DeMan, 1999).

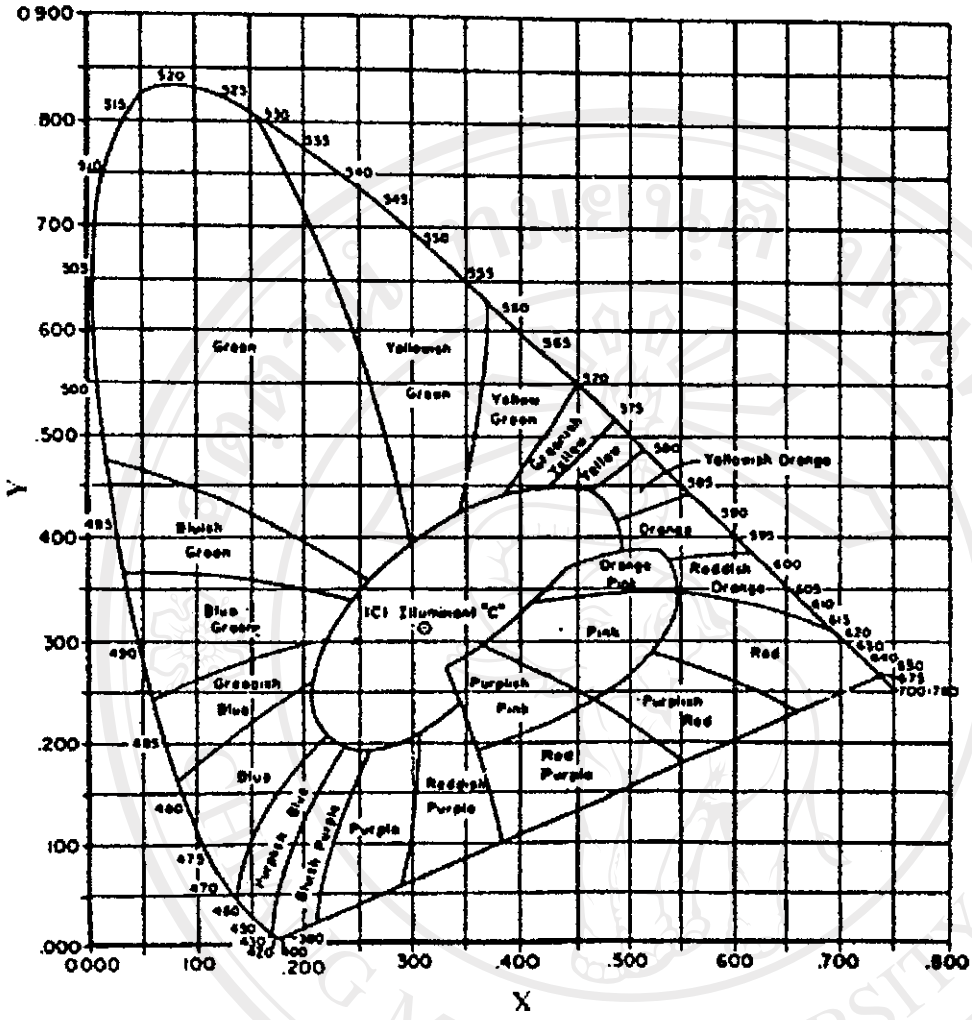

Figure 2 CIE chromaticity diagram (DeMan, 1999).

2.4 Ripe fruit, seed and endosperm.

Inbred seeds of 13 local bitter gourd accessions were planted in the field at PHRC in August 2000. Randomized complete block design (RCBD) with 4 replications was used. Plot size of each accession was 4×6 m^2. Harvesting was started from October 10 to November 8, 2000. Data were collected as follows: -

2.4.1 weight of ripe fruit, weighed the ripe fruit in kilogram.

2.4.2 number of ripe fruit.

2.4.3 weight of seed, weighed the fresh seed in kilogram.

2.4.4 weight of endosperm.

Three replications of 50 g of seed yields of each accession were cracked and their endosperm was separated from seed coat. Weight of endosperm yields in kilogram per rai would be calculated when seeds were harvested. The endosperm of these accessions was used for extraction of 30 kDa protein.

2.4.5 number of days after seed germination to first harvest.

2.4.6 number of days after seed germination to last harvest.

2.4.7 number of harvesting times.

2.4.8 days from anthesis of ripe fruit.

3. Extraction of 30 kDa protein in inbred seeds

Fresh seeds of self-pollinated ripe fruits on the border rows of 13 local bitter gourd accessions with 3 replications were cracked and their seed coats were separated from endosperm. The endosperm of these accessions was stored in a freezer. Methods in extracting and measuring 30 kDa protein from fresh seeds of bitter gourd accessions were conducted at the laboratories of Department of Biochemistry and Medical Science Research Equipment Center Faculty of Medicine, Chiang Mai University between June 6 and November 30, 2000.

3.1 Extraction of partially purifiedly purifiedly purifiedly purified protein.

Partially purified protein was extracted by using modification methods of Lee-Huang *et al.* (1990) and Jiratchariyakul (1999). Five-gram of endosperm from each accession was ground finely with an apothecary's mortar that was surrounded with ice. Fine ground endosperm was put

in a 50 ml beaker (which was surrounded with ice all the time) filled with 20 ml of normal saline solution. These solutions were stirred softly for 15 minutes with a stirrer and adjusted to pH 3.6-4.0 with 1 M hydrogen chloride (HCl). First residue was separated from the solution by filtering through 3 pieces of overlapped cheesecloth. Solution were then spun at 12,000 rpm at 4°C for 30 minutes with a superspeed refrigerated centrifuge (model RC-5). Second precipitate was separated from the spun solution by filtering through a piece of filter paper. Solution was spun again using the same method as described previously. Volume of the supernatant (the clear liquid floating above the precipitate) was measured. This supernatant was mixed together with 16% v/v ammonium sulfate [$(NH_4)_2SO_4$] by using vortex mixer. The supernatant with 16% v/v $(NH_4)_2SO_4$ was called supernate 1 and was spun using the same method as before. After spinning, supernatant that floated above the precipitate was measured and was mixed together with 50% v/v $(NH_4)_2SO_4$ and was called supernate 2 and then was spun in the same way. Precipitate that settled at the bottom of the liquid was mixed together with 50 mM di-sodium phosphate (Na_2PO_4) buffer pH 6.3. This solution at 5 ml was poured into a dialysis bag and dialysed in 200 ml of 20 mM Na_2PO_4 buffer pH 6.3, and stirred with a magnetic bar while incubating at 4°C for 30 minutes. Solution in the dialysis bag was transferred from the old buffer to a new buffer solution (200 ml of 20 mM Na_2PO_4 buffer pH 6.3) and repeated dialysing at 4°C for 12 hour. Partially purified protein fraction in the dialysis bag was poured into a 100 ml flask and dried with a lyophilizer composed of a cooling bath (model CHRIRT K40 FDC-1) and lyophilizer (model ALPHA 1-4) for 24 hours.

Partially purified protein powder of each accession was measured for dry weight and put into tubes. The tubes were sealed before being stored in a freezer.

3.2 Measuring total protein.

Three replications of 5 mg of partially purified protein powder of each local bitter gourd accession was mixed with 1 ml distilled, deionized water (DDI H_2O) and shaken together by vortex mixer. 10 µl of each solution was then piped to microtiter plate wells (model F 96 MAXISORP NUNC-IMMUNO PLATE). Dye reagent was separated by diluting 1 part protein assay dye reagent concentrate (catalog number 500-0006) with four parts DDI H_2O, filter through Whatman #1(or equivalent) to remove particles. Seven dilutions of a protein standard were

prepared and one control (blank) which was representative of the protein solution to be tested. Two samples of protein solutions were assayed per one replication of each local bitter gourd accession. 10 µl of eight levels of concentration of Bovine Serum Albumin (BSA): - 0, 15.625, 31.25, 62.5, 125, 250, 500 and 1000 µg/ml were piped to microtiter plate wells adjacent to the partially purified protein sample. 200 µl/well of diluted dye reagent was mixed with each well of the partially purified protein sample and of the standard protein BSA. The partially purified protein sample and reagent were mixed thoroughly using a microplate mixer. The plunger was depressed repeatedly to mix the solution in each well. The used tips were replaced with clean tips and the reagent was added to the next set of wells. The sample and the standard protein BSA were measured at 620 nm absorbance with spectrophotometer (model (Titertek Multiskan® MCC/340 version 2.33). A regression line of standard protein BSA was calculated. Solution of each accession was compared to standard protein BSA to calculate total protein in the samples from each accession.

3.3 Measuring 30 kDa protein.

Three replications of 5 mg of partially purified protein powder of each local bitter gourd accession was mixed with 1 ml DDI H_2O and shaken together by vortex. Each accession 40 µl of each solution was then mixed with 10 µl of sample buffer and shaken by vortex. Solutions were heated in hot water (95°C) for 10 minutes. 20 µl of each solution was mixed with 5 µl of marker used for running electrophoresis (electrophoresis was run at 150 volt for 90 minutes with a pre run of 100 volt for 10 minutes). Gel sheet was stained ½ hour with 0.1% coomassie blue R-250 and put on a shaker for 12 hours. Gel sheet with protein bands at 30 kDa now visible was repeatedly destained with 40% MeOH/10%HOAc to remove background while on a shaker. Using a slab gel dryer (model SGD 4050) at 70°C. The gel sheet was dried for 2 hours. (model GS-700). Standard proteins with molecular weight at 30, 47 and 67 kDa were used for comparision. The concentration of 30 kDa protein in each sample and standard proteins were determined by imaging densitometer.

4. Extraction of 30 kDa protein in F_1 hybrid seeds.

Two inbred lines of local bitter gourd accessions numbers 13 and 12 which gave the highest yield of 30 kDa protein and two inbred lines of local bitter gourd accessions numbers 10 and 1 which gave the lowest yield of 30 kDa protein (from 3.3) were used in varietal improvement program. They were planted in the field at PHRC on July 26, 2001. RCBD with 2 replications was used. Each accession, plot size was 1×1 m^2. There was one row in each plot and 10 plants in each row. The spacing was 1 m between row and 1 m between plants. Cultural practices were previously described. Each accession was self and cross-pollinated from August 15 to 28, 2001. Diallel crossing system gave 16 treatments (Table 1).

Table 1 Diallel crosses of 4 parental lines of local bitter gourd (16 treatments combination of 4 parents).

Parents (accession number) [1/]	**13**	**12**	**10**	**1**
13	13 × 13	13 × 12	13 × 10	13 × 1
12	12 × 13	12 × 12	12 × 10	12 × 1
10	10 × 13	10 × 12	10 × 10	10 × 1
1	1 × 13	1 × 12	1 × 10	1 × 1

[1/] Parents, accession numbers 13 and 12 gave the first and second highest seed yields, respectively. Accession numbers 10 and 1 gave the first and second lowest seed yields, respectively.

Sixteen treatments of local bitter gourd accessions were harvested between September 4 and 15, 2001. Fresh seeds were cracked and their seed coats separated from endosperm. Five-gram of endosperm from each treatment was ground finely with an apothecary's mortar that was surrounded with ice. Fine ground endosperm was used for extracting and measuring 30 kDa protein at the laboratories of Department of Biochemistry and Medical Science Research Equipment Center Faculty of Medicine, Chiangmai University from October 1 to November 30, 2001.

5. Genetic analysis of 30 kDa protein in F_1 hybrid seeds.

Diallel cross of 4 parental lines of local bitter gourd were planted in RCBD in which there were 16 treatments and 2 replications. If significant *F* ratios occurred in 30 kDa protein of these treatments, the appropriate combining ability analysis would be investigated further. The 4 × 4 table of mean observations were set out as follows: -

♀ Parents \ ♂ Parents	13	12	10	1	Row total $X_{i.}$
13	x_{11}	x_{12}	x_{13}	x_{14}	
12	x_{21}	x_{22}	x_{23}	x_{24}	
10	x_{31}	x_{32}	x_{33}	x_{34}	
1	x_{41}	x_{42}	x_{43}	x_{44}	
Column total X_{j}					Grand total $X..$
					Grand mean $x..$

The following summation notation was used

$$X_{i.} = \sum_{j} x_{ij} = x_{i1} + x_{i2} + x_{i3} + x_{i4},$$

$$X_{j.} = \sum_{i} x_{ij} = x_{1j} + x_{2j} + x_{3j} + x_{4j},$$

$$X.. = \sum_{i}\sum_{j} x_{ij} = x_{11} + x_{12} + \ldots\ldots + x_{43} + x_{44}$$

(all 16 observations).

30 kDa protein of 16 treatments and 2 replications were evaluated for their general combining and specific combining ability from analysis of variance by using Griffing 's method (Griffing, 1956) as follows: -

5.1 The mathematical model for the combining ability analysis was assumed to be

$$x_{ij} = u + g_i + g_j + s_{ij} + r_{ij} + \frac{1}{bc}\sum_k \sum_l e_{ijkl} \quad \begin{cases} i, j = 1, \ldots, p, \\ k = 1, \ldots, b, \\ l = 1, \ldots, c, \end{cases}$$

Where

$i, j = 1, \ldots, p$ = number of parents,

$k = 1, \ldots, b$ = number of blocks(replications) ,

u = population mean,

g_i (g_j) = the general combining ability (GCA) effect for the ith (jth),

s_{ij} = the specific combining ability (SCA) effect for the cross between the ith and jth parents,

r_{ij} = the reciprocal effect involving the reciprocal crosses between the ith and jth parents,

e_{ijkl} = the environmental effect associated with the $ijkl$th.

5.2 The combining ability analysis of variance was given as follows: -

Source of variation	Degree of freedom	Sum of square	Mean square
GCA	p - 1	S_g	M_g
SCA	$p(p\text{-}1)/2$	S_s	M_s
Reciprocal effects	$p(p\text{-}1)/2$	S_r	M_r
Error	m	S_e	M'_e

Where

P = number of parents = 4,

m = number of blocks (replications) × number of plants/block

= 2 × 10

$$S_g = \frac{1}{2p}\sum_i \left(X_{i.} + X_{.i}\right)^2 - \frac{2}{p^2} X_{..}^2,$$

$$S_s = \frac{1}{2}\sum_i \sum_j x_{ij}\left(x_{ij} + x_{ji}\right) - \frac{1}{2p}\sum_i \left(X_{.i} + X_{i.}\right)^2 + \frac{1}{p^2} X_{..}^2,$$

$$S_r = \frac{1}{2}\sum_{i<j} \sum \left(x_{ij} - x_{ji}\right)^2.$$

When M_e is the error mean square for the randomized block design and the M'_e was denoted as

$$M'_e = M_e/m$$

5.3 Testing for overall differences among the various classes of effects were calculated as follows: -

5.3.1 To test GCA effects used

$$F\,[(p-1), m] = M_g / M'_e.$$

5.3.2 To test SCA effects used

$$F\,[p\,(p-1)/2, m] = M_s / M'_e.$$

5.3.3 To test reciprocal effects used

$$F\,[p\,(p-1)/2, m] = M_r / M'_e.$$

The various effects were estimated as follows: -

$$\hat{g}_i = \frac{1}{2p}\left(X_{i.} + X_{.i}\right) - \frac{1}{p^2} X_{..},$$

$$\hat{s}_{ij} = \frac{1}{2p}\left(x_{ij} + x_{ji}\right) - \frac{1}{2p}\left(X_{i.} + X_{.i} + X_{j.} + X_{.j}\right) + \frac{1}{p^2} X_{..},$$

$$\hat{r}_{ij} = \frac{1}{2}\left(x_{ij} - x_{ji}\right).$$