Chapter 3

MATERIALS AND METHODS

Twenty-seven bitter gourd inbred lines were developed from the base population by selfing generation at the Research and Development (R&D) Farm of Lal Teer Seed Ltd., Bashon, Gazipur (Appendix 2). The inbred lines are homozygous for several characteristics. Seven parental lines were selected on the basis of combining ability through line x tester analysis. The correlation and path coefficient analysis were estimated for yield related traits of these inbred. Selected inbred lines were incorporated into diallel fashion for the production of F_1 hybrids to assess their combining ability, heterosis, heritability and selection of the best one hybrid considering all characters of bitter gourd. All experiments were conducted at the Research and Development Farm of Lal Teer Seed Limited at Bashon, Gazipur, Bangladesh during the period of April 2016- April 2019. The general aspects of materials and methods, materials used for the experiments, design of experiments, treatments, methods of raising the crops, data collection and statistical analysis.

3.1 Experimental Site, Soil and Climate

The location of the site is 23.9763^0 N, 090.3539^0 E with an elevation of 8.4 m from the sea level. The soil of the experimental land was clay loam, slightly acidic in reaction (soil p^H 6-6.5, Haider *et al.*, 1991) and poor fertility status under Madhupur tract. The salient feature of the experimental land is given in Appendix 1. The weather data of the growing periods are given in Appendix 3.

3.2 Experiments

The study was aimed at selection of parental lines for diallel crossing, path analysis, analysis of combining ability, variability, heritability and heterosis in bitter gourd. Accordingly, following five experiments were set up:

Experiment I: Evaluation of inbred lines under study

Experiment II: Selection of parents based on genetic back ground utilizing Line x Tester analysis

Experiment III: Genetic analysis of the selected parents based on diallel analysis

Experiment IV: Evaluation of experimental hybrids through heterosis study

Table 3.1 List of twenty-seven inbred lines of bitter gourd used in this study with sources

Entry No.	Inbred lines	Important features	Primary source	Entry No.	Inbred lines	Important features	Primary source
BT-01	TM	Long, smooth, deep green	Thailand	BT-16	Manik WG	Medium, spiny, waxy green	Dhaka
BT-02	TF	Medium, fatty, spiny, green	Dinajpur	BT-17	ANT-200, VLG	Small, very light green	Pakistan
BT-03	TH	Medium, fatty, spiny, more green	Thailand	BT-18	Sakura	Small, blunted, light green	Bogura
BT-04	Chamak-9	Small, light green, fatty	China	BT-19	Prince XL	Long, slender, light green	China
BT-05	ANT-200, DG	Small, cylindrical, deep green	China	BT-20	Peea-1	Small, blunted, green	,,
BT-06	Vishesh, VLG	Medium, light green, less spiny	China	BT-21	Polo-192, G	Small, blunted, green	Thailand
BT-07	Polo VLG	Long, smooth, very light green	Philippines	BT-22	Noree-2	Small, spiny, green	,,
BT-08	ER	Medium, spiny, green	Thailand	BT-23	ER-2	Medium, blunted, green	Thailand
BT-09	TTM	Medium,fatty, spiny,green	LTSL	BT-24	Jessore-4	Medium, spiny, deep green	Jashore
BT-10	PrimeTM, WG	Medium, smooth, green	LTSL	BT-25	Thak-Goz	Medium, very spiny, green	Thakurgaon
BT-11	LOP, LG	Small, smooth, light green	LTSL	BT-26	Chottola-3	Slender, blunted, light green	Chittagong
BT-12	KC	Medium, less spiny, green	Dhaka	BT-27	BK-1	Medium, fatty, green	BARI
BT-13	TK	Medium,light fatty,spiny,green	Thakurgaon				
BT-14	WGT	Medium, very, spiny,waxy green	Thailand				
BT-15	Peea G	Medium,fatty, shiny green	Jashore				

Source: Research and Development Farm, Lal Teer Seed Ltd.

The initial breeding materials for the present research program consisted of twenty-seven bitter gourd inbred lines collected from BARI, Bangladesh, Lal Teer Seed Limited, Bangladesh and foreign countries. The inbred lines were developed by Lal Teer Seed Limited. The materials with their places of collection are listed in Table 3.1.

Experiment-wise materials and detailed methods are mentioned below –

3.2.1 Experiment-I: Evaluation of the inbred lines under study

3.2.1.1 Materials

Initially, twenty-seven bitter gourd inbred lines (listed in Table 3.1) were taken for preliminary evaluation, characterization and selection on yield and yield related traits. These inbred lines were selfed for utilization in the next experiment. The selfing and sib mating program was accomplished by Lal Teer Seed Limited prior to commence the study to maintain the parental lines and to avoid inbreeding depression. Sources of collection of the inbred lines and origin are presented in Table 3.1.

3.2.1.2 Methods

The twenty-seven different inbred lines of bitter gourd were grown in Research and Development Farm of Lal Teer Seed Limited, Bashon, Gazipur during Kharif-1, 2016. The experiment was laid out in RCBD (randomized complete block design) with three replications.

The seeds of the 27 different bitter gourd germplasm were sown in seedling tray. The media in the seedling tray have been prepared by using coconut coir, ash and decomposed cow dung at a ratio of 50%, 25% and 25% respectively (Hasanuzzaman, 2010). The media were boiled by steam for two hours. After cooling the media, the seeds were sown on 27 April, 2016. The seedlings at the age of 2 to 3 leaves were suitable for transplanting and this took 15-20 days after sowing.

The transplantation of seedlings was done on May 12, 2016. Raised beds were prepared for transplanting. The width of raised bed was 1.5 meter. Plant to Plant distance was 1 m and row to row distance was 2 m. Cow dung, Urea, TSP, MP, Insuf, Zinc sulphate and Borax were applied @ 5000kg, 150kg, 100kg, 75kg, 25kg, 8kg and 8 kg per hectare of land respectively. The entire amount of cow dung, TSP, Zinc sulphate, Insuf, Borax and one-third of the urea and MP is applied at the time of final land preparation while the rest of the urea and MP is applied at two equal installments, 20 and 40 days after transplanting (BARC, 2012).

At the time of transplanting, Dursbarn 20 EC and Ridomil MZ 68 WP were used at the rate of 5 ml/L and 3g/L respectively for soil treatment. Irrigation was given as and when necessary. Weeding was done after every 15 days of transplanting to break the soil crust and to keep the plots free from weeds. Trellis was given to the growing plants and allowed them to climb on a rope net.

3.2.1.3 Data collection

Data were collected on the following morphological traits associated with yield from randomly selected plants from each genotype.

3.2.1.3.1 Days to first female flower opening

Number of days was recorded from the date of transplanting to the date when the first female flower opened in a plant. It was recorded from previously selected five plants of each genotype from three replications and the average was calculated.

3.2.1.3.2 Node number of first female flower

The nodes were counted from the lowest to the one at which first female flower opened of all plants and the average was worked out. The cotyledonary leaves were considered as node zero.

3.2.1.3.3 Number of primary branches per plant

The average numbers of branches originating from the main plant were recorded after the plants were pulled out and the average was worked out.

3.2.1.3.4 Days to first harvest

Green matured marketable fruits are harvested and recorded for the first time from each replication.

3.2.1.3.5 Number of fruits per plant

Number of fruits in each plant was counted from ten randomly selected plants and their average number was recorded.

3.2.1.3.6 Single fruit weight

Average weight (g) of ten randomly selected fruits from each replication of genotypes and the average single fruit weight was calculated.

3.2.1.3.7 Fruit length

Fruit length was measured in centimeter (cm) from the neck to blossom end of green fruits. Data were recorded from randomly selected ten fruits in each replication and the average was calculated.

3.2.1.3.8 Fruit diameter

Fruit diameter was measured in centimeter (cm) from along the middle portion of green fruits. Data were recorded from randomly selected ten fruits in each replication and the average was calculated

3.2.1.3.9 Number of seeds per fruit

Average number of seeds per fruit counted from ten randomly selected fruits.

3.2.1.3.10 100- seed weight

Average weight in gram (g) of 100 seeds was obtained from randomly and was weighted by electrical balance.

3.2.1.3.11 Fruit yield per plant

Total number of fruits from selected five plants in the three replications was weighed and their average value was taken in kilogram (kg).

3.2.1.4 Statistical analysis

The data of present study were statistically analyzed. The mean, range, and standard deviation for each character have been calculated and analysis of variance for each of the character was performed by Cropstat 7.2. Genetic parameters like GCV, PCV, heritability and genetic advance were estimated as follows:

3.2.1.4.1 Estimation of genotypic and phenotypic variances

Genotypic and phenotypic variances were estimated according to the formula given by Johnson *et al*. (1955).

Genotypic variance (σ^2 g) = (GMS-EMS)/r

Where, GMS = Mean square of variety

EMS = Mean square of error

r = Number of replications

The Phenotypic variance (σ^2 p) can be calculated by adding genotypic variance (σ^2 g) with Error variance (σ^2 e) as following formula:

Phenotypic variance (σ^2 p) = Genotypic variance (σ^2 g) + Error variance (σ^2 e)

Where, Error variance (σ^2 e) = Error mean square (EMS)

3.2.1.4.2 Estimation of genotypic and phenotypic coefficients of variation

The genotypic and phenotypic coefficients of variation were calculated according to the formula suggested by Burton (1952).

Genotypic coefficient of variation (GCV) = ($\sqrt{\sigma^2 g}$) X 100/Mean

Where, σ^2 g = Genotypic variance

Mean = Population mean.

Similarly,

Phenotypic co-efficient of variation (PCV) = ($\sqrt{\sigma^2 p}$) X 100/Mean

Where, σ^2 p = Phenotypic variance

Mean = Population mean.

3.2.1.4.3 Estimation of heritability

Heritability in broad sense was estimated by the following formula suggested by Johnson *et al.* (1955).

Heritability in broad sense [$h^2(b)$] (%) = (σ^2 g X 100) / σ^2 p

Where,

σ^2 g = Genotypic variance

σ^2 p = Phenotypic variance

3.2.1.4.4 Estimation of genetic advance

The expected genetic advance and genetic advance in per cent of mean were calculated according to the formula used by Allard (1960) and Comstock and Robinson (1952).

Genetic advance (GA) = h^2_b.K. σ p

Where,

h^2_b = Heritability in broad sense

K = Selection differential, the value of which is 2.06 at 5% selection intensity

3.2.1.4.5 Estimation of genetic advance (GA) in percent of mean

Genetic advance in per cent of mean was calculated from the formula given below.

Genetic advance in per cent of mean, GA (%) = (GA X 100)/Mean

Where, Mean = Population mean

3.2.1.5 Selection of yield contributing traits based on trait association

3.2.1.5.1 Estimation of phenotypic and genotypic correlations

Relationships between yield and yield associated traits (thirteen) were studied at genotypic and phenotypic levels. Genotypic and phenotypic correlation coefficients between characters contributing to yield were estimated following the formula suggested by Miller *et al.,* (1958).

Genotypic correlation,

$$r_{g_{1.2}} = \frac{\text{Cov.}_{g_{1.2}}}{\sqrt{V_{g_1} \text{ x } V_{g_2}}}$$

Where,

$\text{Cov.g}_{1.2}$ = Genotypic covariance between the variables X_1 and X_2

Vg_1 = Genotypic variance of the variable X_1

Vg_2 = Genotypic variance of the variable X_2

Similarly, Phenotypic correlation,

$$r_{ph_{1.2}} = \frac{\text{Cov.}_{ph_{1.2}}}{\sqrt{V_{ph_1} \text{ x } V_{ph_2}}}$$

Where,

$\text{Cov.ph}_{1.2}$ = Phenotypic covariance between the variables X_1 and X_2

Vph_1 = Phenotypic variance of the variable X_1

Vph_2 = Phenotypic variance of the variable X_2

3.2.1.5.2 Analysis of path coefficients

The cause-and-effect relationship between yield and its component characters, were studied through path coefficient analysis. Path coefficient of the traits related to yield was analyzed from the phenotypic and genotypic correlations (section 3.2.5.2) following method of Singh and Chaudhary (1985) and Dabholkar (1992) which was originally developed by Wright (1921). Dewey and Lu (1959) employed this method for the first time in plants to short out the direct and indirect influences of component characters on yield in crested wheatgrass. Assuming twelve component characters as independent (X_1, X_2 and X_{12}) and yield as the dependent variable (X_S), path coefficient was obtained by solving a set of simultaneous equations of the following forms:

$$r_{1S} = P_{1S} + r_{12} P_{2S} + r_{13} P_{3S} + r_{14} P_{4S} + r_{15} P_{5S} + r_{16} P_{6S} + r_{17} P_{7S} + r_{18} P_{8S} + r_{19} P_{9S} + r_{110} P_{10S} + r_{111} P_{11S} + r_{112} P_{12S}$$

$$r_{12S} = r_{101} P_{1S} + r_{102} P_{2S} + r_{103} P_{3S} + r_{104} P_{4S} + r_{105} P_{5S} + r_{106} P_{6S} + r_{107} P_{7S} + r_{108} P_{8S} + r_{109} P_{9S} + r_{118} P_{10S} + r_{111} P_{11S} + P_{12S}$$

Where,

$P_{1S}, P_{2S} \ldots P_{12S}$ = path coefficients of the variables $X_1, X_2, \ldots\ldots X_{12}$ on X_S respectively

$r_{1S}, r_{2S} \ldots\ldots r_{12S}$ = correlation coefficients of the variables $X_1, X_2, \ldots X_{12}$ with X_S respectively

The residual effect (R) i.e., the variation unaccounted for by the component characters, which were not studied, was estimated using the formula as follows:

$$\text{Residual effect, } R = [\,1 - (r_{1S} P_{1S} + r_{2S} P_{2S} + \ldots\ldots\ldots\ldots + r_{11S} P_{11S} + r_{12S} P_{12S})]^{½}$$

3.2.2 Experiment-II: Selection of parents based on genetic background utilizing line X tester analysis

3.2.2.1 Materials

Twenty-seven different inbred lines were used to set up a trial of Line x Tester crossing program in Kharif-2, 2016. The seeds were sown on 02 August and transplanted on 20 August, 2016. Out of them 25 lines were used as female and 2 testers were used as male to produce 50 hybrids. All the crosses were made following the appropriate plant breeding technique. The mature F_1 fruits were harvested at maturity stage. The extracted seeds were sun dried and preserved it separately in gene bank at 20^0C and 35% RH with proper label.

From the Line x Tester crossing, a total fifty (50) test F_1's were obtained and preserve it separately for the evaluation in the next year.

In Kharif-1 of 2017, 50 test crosses were studied. The seeds of 50 F_1\`s from Line x Tester cross were sown in February, 2017. Five random samples plants from each plot of replication were taken for data collection.

3.2.2.2 Methods

The seeds of 50 F_1\`s from Line x Tester cross were grown in Research and Development Farm of Lal Teer Seed Limited, Bashon, Gazipur in February 2017. The experiment was laid out in RCBD (randomized complete block design) with three replications.

The seeds of the 50 F_1\`s from Line X Tester cross were sown in seedling tray. The media in the seedling tray have been prepared by using coconut coir, ash and decomposed cow dung at a ratio of 50%, 25% and 25%, respectively (Hasanuzzaman, 2010). The media were boiled by steam for two hours. After cooling the media, the seeds were sown on 26 February, 2017.

The seedlings at the age of 2 to 3 leaves were suitable for transplanting and this took 15-20 days after sowing. The transplantation of seedlings was done on March 18, 2017. Raised beds were prepared for transplanting. The width of raised bed was 1.5 meter. Plant to Plant distance was 1 m and row to row distance was 2 m. Cow dung, Urea, TSP, MP, Gypsum, Zinc sulphate and Borax were applied @ 2kg, 66 g, 92 g, 88 g, 65 g, 1.65 g and 3 g per pit, respectively. The entire amount of cow dung, TSP, Zinc sulphate, Gypsum, Borax and one-third of the urea and MP is applied at the time of final land preparation while the rest of the urea and MP is applied at two equal installments, 15 and 30 days after transplanting (BARC, 2012).

At the time of transplanting, Dursbarn 20 EC and Ridomil MZ 68 WP were used at the rate of 5 ml/L and 3g/L, respectively to protect the seedlings. Irrigation was given as and when necessary. Weeding was done after every 15 days of transplanting to break the soil crust and to keep the plots free from weeds. Trellis was given to the growing plants and allowed them to climb on a rope net.

Data were collected on following morphological and quantitative traits: days to first female flower opening and node number, days of first harvest, fruit length (cm), fruit diameter (cm), single fruit weight (g), number of fruits per plant, branches per plant, number of seeds per fruit, 100-seed weight (g), fruit yield per plant (kg).

3.2.2.3 Analysis of combining ability utilizing line x tester method

In this case the replication wise mean values of F_1 generation of 50 crosses for each trait were subjected to statistical analysis using the following model suggested by Kempthorne (1957) and the solved example given by Dabholkar (1992).

$Y_{ijk} = \mu + g_i + g_j + s_{ij} + e_{ijk}$

Where, Y_{ijk} = value of the ijk^th^ observation of the cross involving i^th^ line and j^th^ tester in k^th^ replication,

μ = general mean (an effect common to all hybrids in all replications),

g_i = general combining ability (GCA) effect of i^{th} line,

g_j = general combining ability (GCA) effect of j^{th} tester,

s_{ij} = specific combining ability (SCA) effect of the cross involving i^{th} line and j^{th} tester,

e_{ijk} = error associated with ijk^{th} observation,

i = i^{th} line (L_1, L_2, L_3, L_4, L_5, L_6 ……. and L_{25}),

j = j^{th} tester (T_1 and T_4), and

k = k^{th} replication (R_1, R_2 and R_3)

3.2.3 Experiment III: Genetic analysis of the selected parents based on diallel analysis

3.2.3.1 Materials

Seven diverse bitter gourd inbred lines were selected on the basis of ranking of parents utilizing by general combining ability (using Line x Tester method) and mean value. These selected seven parents were crossed / hybridized in a diallel mating system including the reciprocals in Kharif-2, 2017. In Kharif-1, 2018, all the seven parents, their 21 F_1s with 21 reciprocal F_1s and Tia as check variety were studied.

3.2.3.2 Methods

The selected seven parental lines were sown in Kharif-2, 02 August, 2017 in seedling tray and transplanted in the field at 20 August, 2017.

Making diallel crosses: Crossing / hybridization was done in 7X7 diallel fashion between the selected diverse and good combiner parental lines. Thus, the crossing combinations were 42 along with 7 parents in this experiment. All the crosses were made following the appropriate plant breeding technique. The mature F_1 fruits along with their respective parents were harvested at maturity stage. The extracted seeds were sun dried and preserved it separately in gene bank at 20^0C and 35% RH with proper label for the evaluation in the next year.

In Kharif-1, 2018, the seeds of seven parents, their 21 F_1's and 21 reciprocal F_1s were sown in seedling tray on 02 March. The experiment was laid out in RCBD (randomized complete block design) with three replications. The media in the seedling tray have been prepared by using coconut coir, ash and decomposed cow dung at a ratio of 50%, 25% and 25% respectively. The seedlings at the age of 3 to 4 leaves were transplanted to field.

The transplantation of seedlings was done on March 20, 2018. Raised beds were prepared for transplanting. The width of raised bed was 1.5 meter. Plant to Plant distance was 1 m and row to row distance was 2 m. Cow dung, Urea, TSP, MP, Gypsum, Zinc sulphate and Borax were applied @ 2kg, 66 g, 92 g, 88 g, 65 g, 1.65 g and 3 g per pit, respectively. The entire amount of cow dung, TSP, Zinc sulphate, Gypsum, Borax and one-third of the urea and MP is applied at the time of final land preparation while the rest of the urea and MP is applied at two equal installments, 15 and 30 days after transplanting (BARC, 2012).

At the time of transplanting, Dursbarn 20 EC and Ridomil MZ 68 WP were used at the rate of 5 ml/L and 3g/L, respectively to protect the seedlings. Irrigation was given as and when necessary. Weeding was done after every 15 days of transplanting.

3.2.3.3 Data collection

Data were collected on days to male and female flower opening and its node number, fruit length, fruit width, fruit weight, number of seeds per fruit, number of fruits per plant, 100 seed weight and yield per plant. Five random samples plants from each plot of replication were taken from F_1 along with their parents for data collection.

3.2.3.4 Data analysis

The data collected on yield and yield contributing characters were subjected to different biometrical analysis to determine variances due to combining ability, heterosis, heritability (broad and narrow sense). Combining ability analysis was carried out following method 1 of model 1 (fixed effect) of Griffing (1956a) using parents, 21 F_1s and 21 reciprocal F_1s populations. In combining ability analysis, the total variance was partitioned into variance due to general combining ability (GCA) effect of the parents and variance due to specific combining ability (SCA) effects of the crosses. The combining ability variances were translated into genetic variance (Bulmer, 1980) and then a heritability value was estimated for each of the characters.

3.2.3.4.1 Analysis of combining ability

The data was analyzed for each character following method-1 and model-1 (fixed effect) as out lined by Griffing (1956a). The model for the combining ability analysis in model 1, method 1 is:

$$Xij = m + gi + gj + Sij + rij + \frac{1}{bc}\sum_k \sum_l e_{ijkl}$$

Where,

i , j = 1, 2, ……………n (number of parents)

k = 1, 2, …………..b (number of blocks)

l = 1, 2, …………..c (number of observation in each plot)

Xij = Mean of I X jth genotypes over k and l

m = population mean

gi = gca effect of ith parent

gj = gca effects of jth parent

Sij = Crosses between the ith and jth parents, i.e., specific combining ability (SCA) effect

r_{ij} = Reciprocal crosses between the ith and jth parents, i.e., reciprocal effect

e_{ijkl} = Environmental effect associated with the ijklth individual observation

$\frac{1}{bc}\sum_k \sum_l e_{ijkl}$ is the Mean error effect.

3.2.4 Experiment IV: Evaluation of experimental hybrids through heterosis study

3.2.4.1 Estimation of heterosis

Heterosis expressed as percent of increase of F_1 hybrid over mid parent (average or relative heterosis), better parent (heterobeltiosis) and Tia F_1 as check variety (standard heterosis) were computed for each character using the following formulae (Fehr, 1987).

a. Heterosis, H (%) over mid parent (MP) = $\frac{\overline{F_1} - \overline{MP}}{\overline{MP}} \times 100$

b. Heterosis, H (%) over better parent (BP) = $\frac{\overline{F_1} - \overline{BP}}{\overline{BP}} \times 100$

Heterosis, H (%) over check (CV) = $\frac{\overline{F_1} - \overline{CV}}{\overline{CV}} \times 100$

Where, $\overline{F}_1$, $\overline{MP}$, $\overline{BP}$ and $\overline{CV}$ are the mean of F_1, two parental populations (MP), better parent (BP) and Tia F_1 as check variety (CV), respectively.

Significant difference of F_1 with MP, BP and CV were tested using 't' test and was calculated by the formula given below:

I. $[t] = \frac{\overline{F_1} - \overline{MP}}{SED}$ x 100 Where, SED = $\sqrt{2EMS/r}$

II. $[t] = \frac{\overline{F_1} - \overline{BP}}{SED}$ x 100 Where, SED = $\sqrt{2EMS/r}$

III. $[t] = \frac{\overline{F_1} - \overline{CV}}{SED}$ x 100 Where, SED = $\sqrt{2EMS/r}$

Where,

SED = Standard error of difference, EMS = Error mean square and r = Replication

SED (Standard error of difference) = √ (Variance/Sample size)