CHAPTER 3

MATERIAL AND METHODS

3.1 Germplasm collection

Twenty-eight commercial rose cultivars which were used for the breeding program appear in Table 3.1.

Table 3.1 Rose cultivars and their details for breeding program

Cultivar name	Colour	Breeder	Type	Flower size
Azure Sea	Silvery lavender	Christensen, Jack E.; Armstrong Nursery	HT	Large
Black Magic	Very dark red	Tantau	HT	Large
Bridal Pink	Light pink	Jackson &Perkins	HT	Large
Dallas	Red	Kordes	HT	Large
Diplomat	Pink	Boerner.	HT	Large
Emblem	Pale yellow	Jackson &Perkins	HT	Large
Emerald	Light peach/green	Preesman	HT	Large
First red	Red	NIRP	HT	Large
Fragrant Cloud	Coral-red becoming geranium –red	Tantau	HT	Large
Frisco	Medium yellow	Kordes	FL	Small
Jade	Light green	Tantau	HT	Medium
Josephine Charlotte	White	De Ruiter	HT	Large
Kardinal	Bright red medium	Kordes	HT	Large
Naomi	Strong pink	Schreurs	HT	Large
Osiana	Light peach/champagne	Tantau	HT	Large
Paris	Deep pink	Meilland	HT	Large
Pink Noblesse	Salmon pink	Tantau	HT	Large
Raphaella	Bright raspberry red/silver outside	Tantau	HT	Large
Ravel	Dark pink	De Ruiter	HT	Large
Saphir	Pink	Tantau	HT	Large
Sundance	Bright yellow	Jackson &Perkins	HT	Large
Texas	Yellow	Kordes	HT	Large
Tineke	White green tinge	Terra Nigra	HT	Large
Top Secret	Red	Meilland	HT	Large
Vendela	Ivory white	Tantau	HT	Large
Vivaldi	Soft pink	De Ruiter	HT	Large
White Noblesse	White	Tantau	HT	Large
Eliza/Persia	Pink	Kordes	HT	Large

Source: Pertwee, 2000; 2003

Pedigree data of parents using in this research were from the Encyclopedia of Rose Science No. 3 (Debener and Gudin, 2003). The genetic relationship of the twenty-eight rose cultivars used in the breeding program was investigated by randomly amplified polymorphic DNA (RAPD) technique and was carried out as follows:

a.) DNA extraction Fresh leaves from rose parents and offspring were sampled separately. The samples were frozen at -20^{o}C till DNA isolation. Each sample was placed in a 1.5 ml micro centrifuge tube and followed by an additional chloroform/isoamyl extraction step, using Doyle and Doyle, 1990. DNA was extracted from 0.1-0.3 g. of fresh frozen leaves and extraction buffer 600 µl containing 4% CTAB, 0.1% β-mercaptoethanol, 20mM EDTA, 1.4M NaCl, 100mM Tris-HCl, 1% PVP. Quantification of DNA was accomplished by analyzing the DNA on 0.8% agarose gel using diluted uncut λ DNA as quantitative standard. DNA was diluted in TE buffer to a concentration of approximately 20 ng/µl for PCR analysis

b.) HAT-RAPD reaction Twenty-eight decamer oligonucleotide primers Kit A (Operon Technologies Inc., California) were screened by polymerase chain (PCR). The following RAPD primer (10-mers) obtained from Operon Technologies (California) were used: OPA-04, OPA-09, OPA-11, OPB-6, OPB-7, OPB-8, OPB-9, OPB-10, OPE-4, OPF-11, OPH-15, OPH-17, OPJ-4, OPN-02, OPN-03, OPN-09, OPN-12, OPO-14, OPP-11, OPR-15, OPT-19, OPW-09, OPX-13, OPAD-01, OPH-01, OPH-03, OPAU-08 and OPR-20. Following the modified protocol of Anuntalabhochai et al., 2000, PCR reactions were performed by using a 20 ml mixture, containing sample DNA (20 ng), 1 x QIAGEN PCR buffer, 100 mM of each dNTP, 10 ng of each primer and 0.5 unit of Taq DNA polymerase.For DNA

amplification, the PCR thermocycler (Perkin Elmer: Gene Amp System 2400) was programmed as follows: incubation at 1 cycle 95°C/5 min; 35 cycles 95°C/45 sec, 48 °C/45 sec, 72°C/1 min and 1 cycle 72°C/5 min. The amplification products were separated by electrophoresis through 1.4% (w/v) agarose gels containing ethidium bromide (0.5 mg/ml) in 1 x TAE buffer and photographed under exposure to UV light using 'Kodak Gel LOGIC 100' digital camera.

c.) Similarity analysis Amplified HAT-RAPD markers as presence or absence of fragment were recorded as 1 (present) or 0 (absent) for each sample. The similarity of sample was calculated as follows:

Similarity = $2 N_{AB}/N_A+N_B$

N_{AB}= the number of bands shared by individuals A and B

N_A=the number of bands individual A

N_B= the number of bands individual B

Phylogenetic relationships among 28 varieties were analyzed in the PAUP program (Version 4.0b10)

3.2 Pairing of parents

3.2.1 Pre-hybridization stage

Pollen germination test were evaluated by pollen dusted onto the artificial medium consisting of 15% sucrose, 100 ppm H_3BO_3, 8.0 g/l of agar, adjusted to pH 5.5-6.5 before boiling, placed in 5 cm Petri dishes (15 ml of medium/dish). Samples of pollen were applied to a medium placed in a petridish to maintain moisture content of pollen grains, using a camel hair brush. The dishes were maintained at 25±2°C under 12 hrs photoperiod provided by cool daylight fluorescent

illumination. After 12 hrs, the pollen grains were examined microscopically and the percentage of germination was determined by counting the number which had germinated (Figure 3.1).

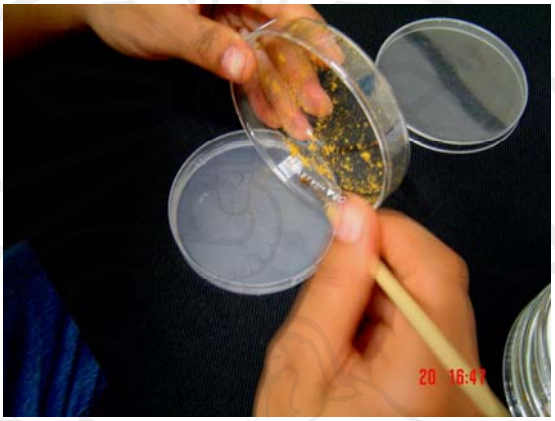

Figure 3.1 Pollen germination test

Pollen releasing, %pollen germination and number of plants were considered for using as pollen source in breeding program.

3.2.2 Hybridization stage

Successful crossing for hip production depended on cross compatibility which resulted in percentage of hip set and fruit drop. The hybridization steps were as follows:

a.) Emasculation: Emasculation was done two days before anthesis. The stage coincided with the development of the flower when the flower bud was almost ready to open. In most hybrid teas and floribundas, this was best done immediately after the petals began to fold back. Because the pollen on some of the multiflora types developed very quickly, therefore removal of the petals might have to be done early, using small forceps before the stamens were visible. All stamens must be removed

using either small scissors or forceps. Occasionally the anthers were entwined in the stigma and care must be taken not to damage the female flower. A paper bag was then placed to cover the emasculated flower.

b.) Pollen preparation: Mature anthers were removed from the flower and stored in a petridish under dry atmosphere to allow the pollen to release from the anther. The pollen was ready for use when it appeared to be floury (yellow, orange and white colour). In some cases, when the anthers were not fully mature, incubation of anthers using incandescent light (100 Watts) for 2 hrs proved to be the best method in terms of pollen releasing time and pollen germination (Figure 3.2).

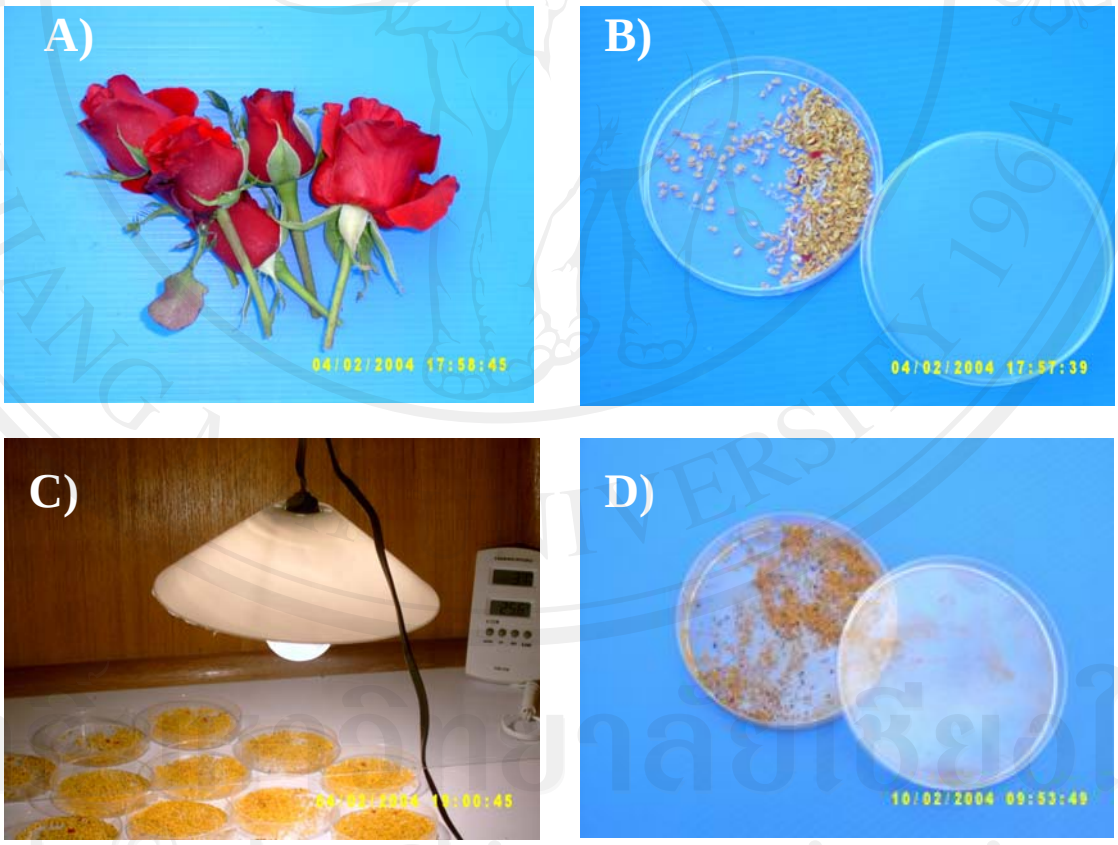

Figure 3.2 Pollen preparation A) blooming stage B) collected anther C) anther incubation D) pollen grains

c.) Pollination: The stigma was considered ready to receive pollen when it appeared to be sticky, about 24 hours after emasculation. Camel brush was used to transfer pollen to the stigma when stigmatic fluid was observed. If several crosses were performed, the brush had to be cleaned in 70% alcohol and allowed to dry thoroughly before they were again used. A single light stroke with a pollen loaded brush was quite adequate (Figure 3.3). If pollination had been successful, the ovary would start to swell in the course of the next few weeks. The fruits would be ripe 3-4 months later.

Figure 3.3 Pollination

d.) Elimination of the side-shoot: The elimination of unwanted side-shoots on the stem bearing flower during hip setting was done by applying 2% $CuSO_4.5H_2O$ as a drop on the axillary buds.

e.) Hip harvesting was done when the hips turned yellow.

f.) Removing seeds from hips:

i) By hand Cut hips into halves or quarters and removed seeds using tweezers. Vinyl or rubber gloves were worn to protect skin, as hips contained thin 'hair' which irritated the skin.

ii) By blender Water was filled in the blender 2 to 3 folds to flood the ripened hips (Figure 3.4) and the blender was allowed to work at the slowest speed, the technique developed by Texas A&M University (2004). Fermentation of the whole content for 2 days proved to be beneficial in order to easily remove the pulp.

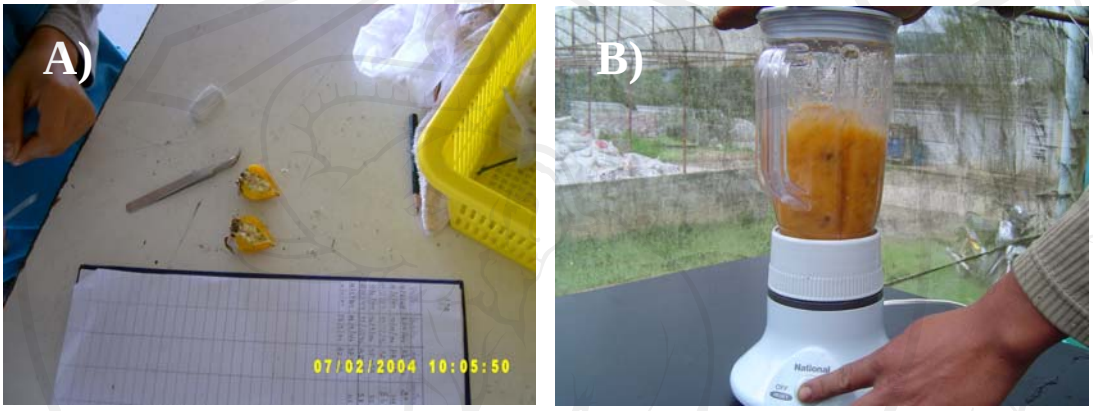

Figure 3.4 Removing seeds from hips

A) by hand B) by blender

g.) Seed cleaning and storage: Cleaning rose seeds was done by 2-3 rounds in clean water after that seeds were dipped in Terraclor solution, 20 ml/20 liters for 5 mins before storage in the refrigerator at 4 ^{o}C.

3.2.3 Post-hybridization stage

a.) Seed stratification: Stratification was done under low temperature by placing the seeds on moist clean peat moss and in cloth bags and kept in the refrigerator for 2 months at 4 ^{o}C. At the end of this cold treatment, radicles of some seeds started to appear and clearly visible.

b.) Seed germination: Seeds were germinated in the sterilized moist sand in plastic baskets burring about 2 cm from surface media with 1 cm thick perlite on top to retain moisture to continue germination process. The seeds were arranged in rows, 1 inch apart. Sprouted seeds were transplanted in plug tray with a ¼ inch top covering with perlite. The plastic tag was placed to describe the parents, date of germination and transplanting date.

c.) Seedling care: Plastic tunnel was used to cover the bench to retain moisture. 40 watt florescent light with cool white spectrum bulbs were placed 40 cm above the seedling tray. Lighting was on for 5 hours before day-break. Before the first blooming, seedlings were transplanted into 4 inch pots filled with sterilized potting media (peat moss). Perlite was again added on the top. Seedlings at cotyledon stage were transplanted to a new media. A solution of Terraclor (20ml/20 liters of water) was given from time to time to prevent damping off disease. The plants were watered by flooding technique through the bottom part using rain water. Nutrient solution CMU-RPF (1:400) was given regularly together with hormone solution (Rootgro 1 ml/ 20 liters of water) every week. Young plants were again transplanted into 6-inch pots with the same potting media.

3.3 Selection

Evaluation criteria were as follows:

3.3.1 Seedling stage: Discard criteria as follows: abnormal seedling, slow growth and diseased.

3.3.2 Small plant size stage (4-inch pot): The characteristics of first, second and third flowers were examined for bud size, flower diameter, number of petals, number of leaves per stem and stem length.

3.3.3 Medium plant size stage (6-inch pot): Bud size, size of opened flower, peduncle diameter, peduncle length and peduncle/bud size ratio were considered.

3.3.4 Large plant size stage (12-inch pot): Selection criteria were as follow: good branching, good vigor, floriferousness and attractiveness of foliage, flower colour, number of blooms, number and texture of petals and good quality of stems. The size of the flowers and their relation to the stem was also evaluated.

3.3.5 First budding stage: Growth type, number of petals, diameter of bud union, proportion of neck length (peduncle /flower height), basal shoot at the position of bud union (6 months), plant height, bud size and flower diameter were considered.

The various anatomical parts of a rose (sepal, petal, calyx, thorn, bract, peduncle, foliage, etc.) provided the verification.

1. Bloom form

   -cut stage of possible beauty, formal shape, circular outline and symmetry

   -well-proportioned (well-balanced/arranged/formed/center, pointed/high-centered, regularity in shape and good petal placement and number of petals)

2. Bloom colour

   -brightness, freshness, clarity, purity and brilliance

3. Petal substance

-texture, crispness, thickness, firmness, stiffness and stability of colour

4. Bloom size
5. Stem and foliage
   - stem: well proportioned to flower and foliage (strong stem, good stem length, straightness, size, and few thorns, balance and proportion to foliage and bloom)
   - foliage: well placed and spaced in proportion to stem and flower (glossiness, leaf size, leaf characteristics)
6. Balance and proportion
   - neck length: (proportion of peduncle and flower length)

3.3.6 Second budding stage: Outstanding plants with desired characters were selected and evaluated on the following characters: productivity, flush and yield trial. At this stage the budded plants were selected with an emphasis on disease resistance, bending peduncle and high yield. For the screening for yield and quality, seven characters i.e. number of strong canes, number of flower/plants, flush, strong peduncle, petal injury, flower opening and split center, were considered. The scoring method was presented in Table 3.2.

Table 3.2 Scoring method for yield and quality of roses

Score	No. of strong cane	No. of flower per plants	Flush	Strong Peduncle	Petal Injury	Flower Opening	Split Center
1	<5	<9	>66	weak	all	very rapid	all
2	5-6	10-11	61-65	rather weak	almost all	rapid	almost all
3	7-8	12-13	56-60	intermediate	some	intermediate	some
4	9-10	14-15	50-55	strong	few	slow	few
5	>11	16-17	<50	very strong	none	very slow	none

3.3.7 Grouping of colour

Selected numbers with various colours were classified, grouped and compared with the commercial standard varieties.

3.4 Inheritance of parents

3.4.1 Cytological study Root tips of 1-2 cm were collected from seedlings showing vigorous growth, washed with water and fixed in fixative solution consisting of absolute ethanol : acetic acid = 3:1(v/v), 30-45 mins. Fixative should be freshly prepared before use. Root tips were then rinsed and placed in test tubes. A sufficient amount of 1 N HCl was applied and heated at 60 ^{o}C for 3-5 min, followed by rinsing twice with water. Cut root tips 1-2 mm below root apex on glass slides. Put a drop of aceto-orcin on the slide, squashed and apply a cover slip after that the slide was exposed briefly over flame. Wiped the edge of the slide to remove excessive stain and observed slide under microscope. Chromosome number of 10 well-spread at metaphase stage were counted under a microscope and photographed.

3.4.2 DNA fingerprints DNA was extracted from fresh leaves by procedure of Doyle and Doyle (1990) using high annealing temperature random amplification of polymorphic DNA (HAT-RAPD) technique of Anuntalabhochai *et al.*, (2000). The primers were tested as single primers for their ability to amplify rose between parents and their offspring.

3.4.3 Heritability Narrow-sense heritability were estimated by Kearsey and Pooni (1996) method and analysis by Falconer and Mackay (1996)

3.5 Yield trials

Red and pink offspring were evaluated in the greenhouse. The comparisons between offspring (6 selected numbers) and commercial cultivars (1 variety) were conducted. CMURPF mix media, containing coco peat, rice husk and sand (6:3:1, v/v/v) in plastic baskets was used as a medium. The plants were watered by automated timed drip irrigation system. There were six plants in each container. Cultural practices and harvesting techniques were uniformly applied to all plants according to the commercial practices. During each season, all plants were lightly pruned, taking out non-productive and weak growth stems.

Shoots with peanut-sized flower bud were bent using arching cultivating technique by making a kink at the base of the shoot. Subsequently, short or blind shoots were also bent weekly throughout the experiment. Flower buds of the bent shoots were removed before bending took place.

Bud size, stem length and number of harvested flowering shoots per experimental unit were measured on each flush to assess the productivity and quality of cut-flower roses for rainy seasons, winter and summer based on Royal Project

Foundation standardized grading (Table 3.3). Flowering shoots were deemed harvestable when at least one sepal of the flower was horizontally reflexed. Stem length of flowering shoots was measured from the shoot base to the base of flower bud before harvesting. Records were kept during in 2007-2008.

Table 3.3 Standardized grading for cut-rose of Royal Project Foundation

Parameter	Extra	1	2	3	U
Flower diameter (cm.)	>4	3.5	3	2.5	<2.5
Stem Length (cm.)	70	60	50	40	<40

Source: Royal Project Foundation, 2008.

Each selected numbers were evaluated on the following characters for overall plant performance (Table 3.4), quality (Table 3.5) and yield (Table 3.6):

Table 3.4 Selection criteria for overall plant performance

Plant Characters (25 points)							
Bush :	Tall	5	4	3	2	1	Short
Side shoots:	Very few	5	4	3	2	1	Very many
Pickles:	Very few	5	4	3	2	1	Very many
Stem length[1]:	Long	5	4	3	2	1	Short
Bud union[1]:	Big	5	4	3	2	1	Small
Problem on Field (25 points)							
Spiral center opening:	Spiral	5	4	3	2	1	Split
Flower opening:	Normal	5	4	3	2	1	Abnormal
Fading flower:	Clear	5	4	3	2	1	Discoloured
Strong peduncle:	Strong	5	4	3	2	1	Weak
Flower shape:	Attractive	5	4	3	2	1	Poor
Overall Flower Characters (60 points)							
Bud stage:	Attractive	5	4	3	2	1	Poor
Semi-opening stage:	Attractive	5	4	3	2	1	Poor
Opening stage:	Attractive	5	4	3	2	1	Poor
Brightness of colour:	High	5	4	3	2	1	Low
Cleanness of uniform colour:	High	5	4	3	2	1	Low
Petal thickness:	Thick	5	4	3	2	1	Thin
Petal texture:	Smooth	5	4	3	2	1	Rough
Straightness of stem:	Straight	5	4	3	2	1	Curvy
Glossiness of leaves:	Glossy	5	4	3	2	1	Dull
Proportion stem/leaves	Balance	5	4	3	2	1	Unbalance
Size of flower[1]:	Big	5	4	3	2	1	Small
Number of petals[1]:	High	5	4	3	2	1	Low
Productivity Trends (60 points)							
Uniformity of sprouting:	Uniform	5	4	3	2	1	Poor
Sprouting after bending:	Good	5	4	3	2	1	Poor
Sprouting after harvesting:	Good	5	4	3	2	1	Poor
Plant building:	Good	5	4	3	2	1	Poor
Levels to propagation:	Easy	5	4	3	2	1	Difficult
Stability of bent shoot:	Stable	5	4	3	2	1	Broken
Transport:	Good	5	4	3	2	1	Poor
Flush to flush[1]:	Short	5	4	3	2	1	Long
Productivity[2]:	High	5	4	3	2	1	Low
>%CK[2]:	Higher	5	4	3	2	1	Lower
%High/ Low grade[1]:	Good	5	4	3	2	1	Poor
Vase life[1]:	Long	5	4	3	2	1	Short

Note: 1=see Table 3.6, 2=see Table 3.7

Table 3.5 Selection criteria for quality

Score	Size of flower	%High/ low grade	Vase life	Flush	Stem length	Bud union size	Number of petals
1	<2.0	0-10/100-90	<7	>66	<50	1.1-2.0	<15
2	2.1-2.5	11-20/80-89	7-9	61-65	51-70	2.1-3.0	16-25
3	2.6-3.0	21-30/70-79	10-12	56-60	71-90	3.1-4.0	26-35
4	3.1-3.5	31-40/60-69	13-15	50-55	91-100	4.1-5.0	36-45
5	3.6-4.0	41-50/50-59	>15	<50	>100	>5.0	>46

Table 3.6 Selection criteria for yield

Score	%CK	Productivity
1.0	<-11	<160
1.5	-10--1	161-180
2.0	0-10	181-200
2.5	11-20	201-221
3.0	21-30	221-240
3.5	31-40	241-260
4.0	41-50	261-280
4.5	51-60	281-300
5.0	>60	>300

Analysis of variance was used to compare the mean number of flowering stems and the total number of harvested and unmarketable stems between cultivars and between seasons. Data were presented as mean or totals for each season (four months).Statistical analyses were done using MSTATC, Excel 2007 and STATISTICA version 8.0.

3.6 Market response trial

a.) General public The objectives of this survey group were to study the response on perception of consumers to the new rose cultivars. The questionnaires, developed from the focus group report, consisting of 2 parts: the consumer attitude to behavior of the new rose cultivars and personal data of the consumers of roses in general.

b.) Florist or flower shop The procedure was the same as the previous group.

The 9-points hedonic scale was used to measure product acceptance and preference and the scale categories corresponded to 9=like extremely, 7=like very much, 5= like moderately, 3= like slightly and 1= neither like nor dislike. These values were assigned to the attribute of consumer desire trait for beauty of flower, bud shape, attractiveness of colour, texture of petals, stems and leaves, proportion and balance, size of flower, scent, thorn and overall acceptability.

The questionnaire was coded to facilitate data entry. The data were analyzed using the MSTATC software version 3. The percentages of responses of the personal data and attitudinal data were calculated. Differences were regarded as significant at $P \leq 0.05$.