CHAPTER 3

MATERIALS AND METHODS

Plant materials

Seven pea lines/cultivar comprised of 3 powdery mildew resistant lines, P117, P185 and P309 and 4 commercial powdery mildew susceptible lines/cultivar, No.3, No.4, No.5 and Fang No.7, were used as a germplasm in this breeding program. These pea lines/cultivar were obtained from 3 sources (Table 3.1).

Table 3.1 Sources of pea lines/cultivar, types and powdery mildew phenotypes

Line/ Cultivar	Source	Type	Powdery mildew phenotype
P117	Department of Agriculture	Garden pea	Resistant
P185	Department of Agriculture	Garden pea	Resistant
P309	Department of Agriculture	Semi-snow pea	Resistant
No. 3	China	Snow pea	Susceptible
No. 4	China	Snow pea	Susceptible
No. 5	China	Snow pea	Susceptible
Fang No.7	commercial cultivar	Snow pea	Susceptible

3.1 Morphological characteristics, yield evaluation and seed multiplication

Seven pea lines/cultivar as mentioned above were planted in the field conditions at Pang Da Royal Agricultural Station, Samoeng district, Chiang Mai in the winter season during the period of November 2006 to February 2007. Randomized Complete Block Design (RCBD) was used with 6 replications, 10 plants per each

replication. After transplanting, plant growth, heights, number of nodes and branches were recorded. The other variables like yield performances, pod widths and lengths, seeds per pod, were also collected. The Statistix program SXW version 8.0 was used for data analysis.

Due to less number of seeds per lines/cultivar, seeds multiplication was also conducted by allowing self-pollination and seeds from each line/cultivar were harvested for further experiment.

3.2 Phenotypic evaluation of powdery mildew resistance

Evaluation of powdery mildew resistance on seven pea lines/cultivar was carried out in two locations, Pang Da Royal Agricultural Station and Inthanon Royal Agricultural Research Station. The details of both evaluations are as follows:

3.2.1 Field evaluation

The field condition testing was done at Pang Da Royal Agricultural Station where is located at the height about 700 meters above sea level, in winter season during November 2006 to March 2007.

3.2.2 Greenhouse condition

This procedure was conducted at Inthanon Royal Agricultural Research Station, JomThong district, Chiang Mai where is located at height about 1,200 meters above sea level, in rainy season during July to October 2007.

In both sites, the similar trial procedures were achieved. Fourteen days prior to transplanting, the susceptible cultivar Fang No.7 was grown as source for *Oidium* spp. inoculum or spreader rows. Randomized Complete Block Design RCBD

with 6 replications was used. All plants of seven pea lines/cultivar, P117, P185, P309, No.3, No.4, No.5 and Fang No.7, were transplanted on the same day. Evaluations were done in according to the presence of disease at the 4^{th}, 7^{th}, 10^{th} nodes and on a whole plant at 45, 55, 65 and 75 days, respectively. Infected leaves areas were measured using the method as described by Young *et al.* (1993) and Ondrej *et al*. (2003) (Table 3.2). Disease severity scoring was based on the amount of disease on each leaf and calculated for the percentage of infected area ranging from 0 to 100% (Figure 4.1) and then they were analyzed by Statistix SXW version 8.0.

Table 3.2 Classification scale of powdery mildew disease severity group on Young *et al.* (1993) and Ondrej *et al*. (2003)

Percentage of leaf infection	Disease reaction	Group classification
0	Highly resistant	Resistant group
1-25	Resistant	
26-50	Moderate resistant	Moderate resistant group
51-75	Moderate susceptible	
76-100	Highly susceptible	Susceptible group

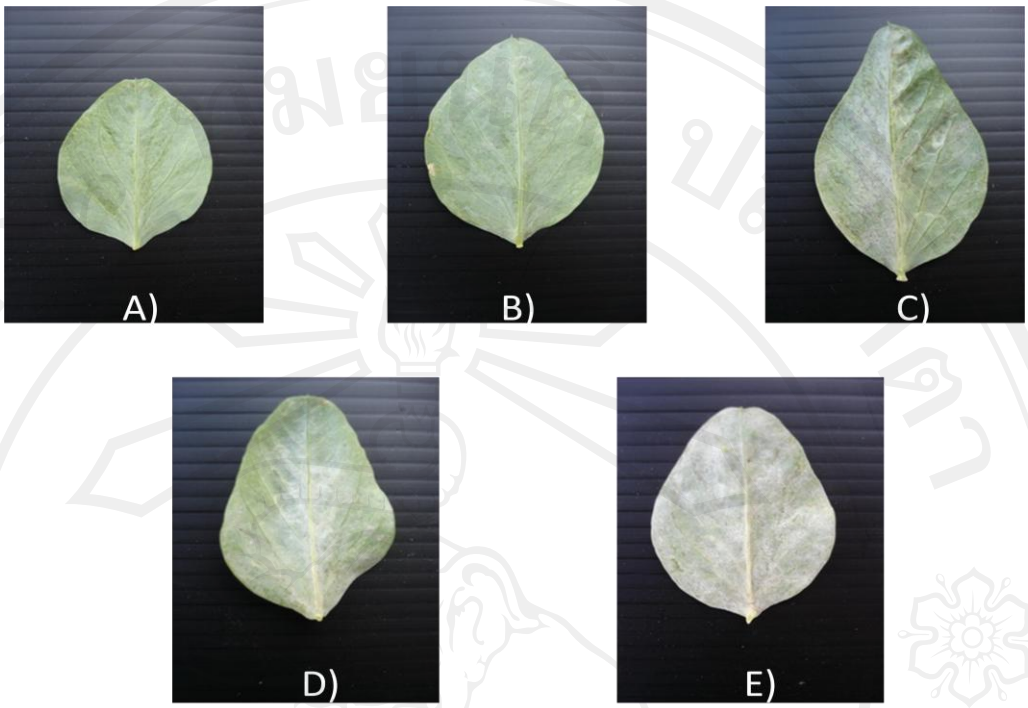

Figure 3.1 The percentage of powdery mildew infection area on pea leaves

A) 0% B) 1-25% C) 26-50% D) 51-75% E) 76-100%

3.3 DNA marker linked to powdery mildew resistance in snow pea

3.3.1 Suitable primer screening

Three primers OPU-17, OPO-02, and ScOPD-10, and PCR protocol for DNA marker linked to powdery mildew resistant and susceptible gene in pea described by Janila and Sharma (2004) were used. Seven pea lines/cultivar which were used in this breeding program comprised of 3 resistant lines, P117, P185 and P309, and 4 susceptible to powdery mildew lines No.3, No.4, No.5, and cultivar Fang No.7, were tested to identify their resistance by using polymerase chain reaction (PCR) technique (Table 3.3).

Table 3.3 Primer types, sequence and linkage DNA marker position reported by Janila and Sharma (2004)

Primer	Primer type	Primer sequence (5´ - 3´)	Linkage DNA marker position
OPU17	Random primer	-ACCTGGGGA-	1000 bp. in resistant plant
OPO02	Random primer	-ACGTAGCGTC-	1400 bp. in susceptible plant
ScOPD10	SCAR primer	(f) -GGTCTACACCTCATATCTTGATGA- (r) -GGTCTACACCTAAACAGTGTCCGT-	650 bp. in resistant plant

3.3.1.1 DNA extraction

Fresh young pea leaves of each line/cultivar were sampled and cleaned separately. Each 0.3 g of sample was pulverized with 0.3 ml of Plant DNAzol® reagent (Invitrogen co., ltd.). Then, they were placed in 1.5 ml of microcentrifuge tube and incubated at 25˚C with shaking for 5 min, after that they were added with 0.3 ml of chloroform, vigorously mixed and continuously incubated at 25˚C with shaking for 5 min. Next, the extract was centrifuged at 10,000 rpm for 10 min. After extraction, the supernatant was transferred to a new tube and centrifuged again. The DNA was precipitated by mixing the aqueous phase with 0.225 ml of 100% ethanol. After addition of ethanol, samples were mixed by inverting the tubes 6 to 8 times and stored them at room temperature for 5 min. DNA sediment was precipitated at 5,000 rpm for 4 min then, the resulting supernatant was removed.

Plant DNAzol-ethanol wash mixture was prepared by mixing 1 volume of Plant DNAZOL with 0.75 volume of 100% ethanol. Plant DNAzol-ethanol wash solution, 0.3 ml, was mixed with the precipitated DNA by vortexing. The samples were stored for 5 min and centrifuged at 5,000 rpm for 4 min. Then,

DNAZOL wash solution was removed and the DNA pellet was washed with 0.3 ml of 75% ethanol followed by centrifugation at 5,000 rpm for 4 min. Ethanol wash was removed by decanting and the tubes were stored as vertically for 1-2 min and then removed the remaining ethanol and dried the DNA pellet. The DNA pellet was dissolved in TE buffer (pH 8.0). Quantification of DNA was accomplished by analyzing the DNA on 1.0% agarose gel using 100 bp Sharp DNA Ladder Marker (RBC Bioscience Inc., Taiwan) as quantitative standard. DNA was diluted in TE buffer to 15 ng/µl for PCR analysis.

3.3.1.2 PCR technique

Two decamer oligonucleotide primers, RAPD primers (10-mers) OPO-02 and OPU-17, and one SCAR primer, ScOPD-10, from Operon Technologies Inc. (Alameda, California, USA) were used to screen for PCR polymorpism. Reactions for all PCRs were according to the procedure of Janila and Sharma (2004). Each 20 µl sample of PCR reaction mix contained 15 ng of DNA template, 1× PCR buffer (10mM Tris-HCl pH 8.3, 50mM KCl, 0.2 mM $MgCl_2$, Enhancer solution), 0.5 mM of each dNTPs, 80 ng of a given primer, 1 unit of *i-Taq*^{TM} DNA polymerase (iNtRON Biotechnology, Inc.) and dH_2O. PCR reactions were performed in the Perkin Elmer Gene Amp PCR System 2400 (Perkin-Elmer Cetus Co.) programmed for one cycle at 92 ◦C for 2 min, followed by 44 cycles at 92 ◦C for 30 sec, 42 ◦C for 30 sec and 72 ◦C for 1 min and a final extension at one cycle at 72 ◦C for 5 min. PCR products were resolved using 1.7% agarose gels in 1× TBE buffer by electrophoresis. The amplified DNA bands were stained in 0.1 µg/ml of ethidium bromide. Then stained gels were visualized on UV light transilluminator

and photographed using BIODOC-It ™ M-20 System (Gibthai Co., Ltd.). The marker position was detected by comparing with 100 bp and 1.5 Kb DNA ladder.

3.3.2 DNA sequence analysis

The specific polymorphic DNA bands for powdery mildew resistance in pea lines were identified. The specific DNA bands were extracted from the gel and used for reamplification by the same primer using selective amplification condition. The fragments were then cloned with Clone Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience Co., Ltd.). After purification, DNA was sequenced at Pacific Science Co., Ltd. Then, the DNA sequences were compared with database in National Center for Biotechnology Information (NCBI) GenBank.

3.4 Hybridization for powdery mildew resistance

3.4.1 Crossing and selection

Three powdery mildew resistant lines, P117, P185 and P309, and 4 powdery mildew susceptible lines/cultivar, No.3, No.4, No.5, and Fang No.7, were planted for crossing. Reciprocal crosses were made among all resistant and susceptible lines/cultivar in the greenhouse conditions at Pang Da Royal Agricultural Station in winter season during November 2006 to March 2007. Twenty-four crosses including reciprocal cross were made (Table 3.4).

Table 3.4 Parental lines/cultivar and crosses including reciprocal crosses of powdery mildew resistant lines and susceptible lines/cultivar

Resistant line	Susceptible line/cultivar			
	No.3	No.4	No.5	Fang No.7
P117	P117 × No.3	P117 × No.4	P117 × No.5	P117 × Fang No.7
	No.3 × P117	No.4 × P117	No.5 × P117	Fang No.7 × P117
P185	P185 × No.3	P185 × No.4	P185 × No.5	P185 × Fang No.7
	No.3 × P185	No.4 × P185	No.5 × P185	Fang No.7 × P185
P309	P309 × No.3	P309 × No.4	P309 × No.5	P309 × Fang No.7
	No.3 × P309	No.4 x P309	No.5 × P309	Fang No.7 × P309

F_1 hybrids of all crosses were planted in the greenhouse at Inthanon Royal Agricultural Research Station during the period of April to July 2007. Pod characteristics of each cross were evaluated for the desirable characters which were similar to edible snow pea pods. Due to resistance to powdery mildew controlled by recessive single *er* gene (Janila and Sharma, 2004), all F_1 generations were completely susceptible genotype. Each plant was then allowed to have a self-pollination to make F_2 hybrids for further evaluation.

Twenty-three F_1 reciprocal cross hybrids from 3.4.1 (cross between No.3 × P309 plants died when they were at seedling stage) were screened to identify for the suitable crosses which had the closely desirable characteristics to recurrent parent. Only four crosses of F_2 generation derived from crosses between No.3 × P309, P309 × No.4, No.5 × P309 and Fang No.7 × P309 were used for backcrossing.

3.4.2 Backcrossing

In this breeding program, three backcrossing generations were planned to increase the desirable trait from recurrent parent to new cultivar. Due to the limitation of trial area, cultivar improvement of four selected F_2 hybrids which derived from crosses between No.3 × P309, P309 × No.4, No.5 × P309 and Fang No.7 × P309 by backcrossing method was divided into two groups, group I, Fang No.7 × P309 and group II, No.3 × P309, P309 × No.4 and No.5 × P309.

3.4.2.1 The first backcrossing

a) Cross of Fang No.7 × P309

Backcrossing of this cross was carried out in the greenhouse condition at Inthanon Royal Agricultural Research Station during the period of August to November 2007. Fourteen days before transplanting of F_2 plants, the susceptible cultivar, Fang No.7, was grown as the spreader rows for identifying the resistant and susceptible F_2 plants. At 10 days after transplanting, F_2 plants were evaluated. The powdery mildew resistant plants which had pod width more than 2.0 cm were chosen.

The resistant F_2 plants in the field condition were confirmed for their resistance by using DNA maker linked to powdery mildew resistant trait followed in topic 3.3.1 by using PCR technique. The resistant F_2 plants which showed DNA marker band were selected. Due to Fang No.7, commercial cultivar, which was used as female (recurrent parent), was heavily infected with powdery mildew, thus selected F_2 plants were used as female (recurrent parent) instead of cultivar Fang

No.7 which was used as male (donor parent) to produce BC_1 generation. BC_1F_1 seeds were collected for the next planting.

BC_1F_1 hybrids which were developed at Inthanon Royal Agricultural Research Station were planted for self-pollinated in order to produce BC_1F_2 seeds in the greenhouse condition at Pang Da Royal Agricultural Station during the period of December 2007 to March 2008. At maturity stage, BC_1F_2 seeds were collected for the second backcrossing.

b) Cross of No.3 × P309, P309 × No.4 and No.5 × P309

The F_2 hybrid seeds which derived from 3 crosses between No.3 × P309, P309 × No.4 and No.5 × P309 were planted for powdery mildew resistant phenotypic evaluation in the greenhouse condition at Pang Da Royal Agricultural Station during the period of December 2007 to March 2008. Fourteen days prior to transplant F_2 plants, the susceptible cultivar, Fang No.7, was grown as *Oidium* spp. host inoculums rows for identifying the resistant and susceptible F_2 plants. At 65 days after transplanting, a whole plant of all grown F_2 generation was evaluated for disease resistance following the method as described in 3.2. The pod width more than 2.0 cm of powdery mildew resistant plants was chosen.

The resistant F_2 plants in the field condition were confirmed for their resistance by using DNA maker linked to powdery mildew resistance trait followed the topic in 3.3.1 by using PCR technique. The resistant F_2 plants which appeared DNA marker band were chosen. Due to lines No.3, No.4 and No.5 which were used as recurrent parent, were heavily infected with powdery mildew, thus selected F_2 plants which derived from cross between No.3 × P309, P309 × No.4 and

No.5 × P309 were used as female (recurrent parent) while lines No.3, No.4 and No.5 were used as female.

BC_1F_1 seeds of each cross were collected for the next planting cycle which was planted for self-pollinated in order to produce BC_1F_2. This process was conducted in the greenhouse condition at Inthanon Royal Agricultural Research Station during the period of April to July 2008. After pod maturity, BC_1F_2 seeds were collected for the second backcrossing.

3.4.2.2 The second backcrossing

a) Cross of Fang No.7 × P309

Obtained BC_1F_2 seeds from first backcrossing 3.4.3.1 were planted in the greenhouse condition at Inthanon Royal Agricultural Research Station during the period of April to June 2008. Fourteen days prior to transplant BC_1F_2 plants, the susceptible cultivar, Fang No.7 was grown as *Oidium* spp. host inoculums rows for identifying the resistant and susceptible BC_1F_2 plants. At 48 days after transplanting, BC_1F_2 plants were evaluated. The pod of powdery mildew resistant plants which was greater than 2.0 cm were chosen.

The resistant BC_1F_2 plants in the field condition were confirmed for their resistance by using DNA maker linkage to powdery mildew resistance trait followed topic 3.3.1 in PCR technique. The resistant BC_1F_2 plants which showed DNA marker band were selected as female to cross with cultivar Fang No.7 as male. Due to BC_2 backcross generation was not successful for two times, BC_1F_2 and BC_1F_3 were allowed self-pollinated, finally, the second backcrossing, BC_1F_4 was used to cross back as a reciprocal method with the recurrent parent, cultivar Fang No.7 at

Inthanon Royal Agricultural Research Station during the period of November 2008 to February 2009. BC_2F_1 seeds were planted for self-pollinated in order to produce BC_2F_2 seeds in the greenhouse at Phamon village, Inthanon Royal Agricultural Research Station during March to June 2009 for producing BC_2F_2 by self-pollination. BC_2F_2 seeds were collected for the third backcrossing.

b) Cross of No.3 × P309, P309 × No.4 and No.5 × P309

The BC_1F_2 seeds were grown in greenhouse at Inthanon Royal Agricultural Research Station in summer during August to November 2008 under the same condition as described in 3.4.3.1b). The screening for powdery mildew resistantce and good agricultural traits in BC_1F_2 plants were processed. The resistant plants which had large pod and good agricultural traits were chosen.

The resistant BC_1F_2 plants in the field conditions modifying circumstance were confirmed for their resistance by using PCR technique with the same primer and condition as mentioned above in 3.4.2.1 b). Then confirmed for resistant BC_1F_2 plants with DNA fingerprint were used as female parent to cross with susceptible to powdery mildew disease lines which was used as male. The BC_2F_1 seeds were collected. They were grown and self-pollinated for producing BC_2F_2. This step was processed in the greenhouse conditions at Pang Da Royal Agricultural Station during the period of December 2008 to March 2009. At the maturity stage, BC_2F_2 seeds were collected for the third backcrossing.

3.4.2.3 The third backcrossing

a) Cross of Fang No.7 × P309

The BC_2F_2 seeds from 3.4.2.2 were grown for powdery mildew plant evaluation in the greenhouse at Inthanon Royal Agricultural Research Station during the period of June to August 2009. The selection of powdery mildew resistant and good agricultural traits in BC_2F_2 plants were processed as described in 3.4.2.1 a). The resistant plants which had large pod and good agricultural traits were chosen.

The resistant BC_2F_2 plants in the field were confirmed for their powdery mildew resistance by using PCR technique followed the method as described in 3.4.2.1 b). Confirmed resistant BC_2F_2 plants were used as female to cross with the susceptible to powdery mildew disease cultivar, Fang No.7, which was used as male. The obtained BC_3F_1 seeds were grown for self-pollinated in order to produce BC_3F_2. This step was processed in the greenhouse condition at Inthanon Royal Agricultural Research Station during the period of August to November 2009. Then, BC_3F_2 seed were screened for the resistant plants in the field condition at Pang Da Royal Agricultural Station during November 2009 to February 2010. Those of them were confirmed for their resistance by using DNA maker with the same primer and protocol as to be described in 3.3.1. Confirmed resistant BC_3F_2 plants were allowed self-pollinated to produce BC_3F_3 seeds. Then, they were tested for powdery mildew resistance and yield in other Royal Project pea production areas.

b) Cross of No.3 × P309, P309 × No.4 and No.5 × P309

BC_2F_2 plants were grown in the field condition at Inthanon Royal Agricultural Research Station during the period of July to September 2009 and they

were evaluated for powdery mildew resistance and good agricultural traits as the same process and condition that mentioned above. Confirmed resistant gene was also processed as the same method as to be described in 3.4.3. The confirmed resistant BC_2F_2 plants were used as female to cross with commercial lines, No.3, No.4 and No.5, which were used as male. The BC_3F_1 hybrid seeds were collected and they were then planted for producing self generation seed (BC_3F_2) at Inthanon Royal Agricultural Research Station during the period of October to December 2009. The BC_3F_2 plants were screened the resistant plants at Pang Da Royal Agricultural Station during the period of December 2009 to March 2010. Those of them were allowed self pollinated to produce BC_3F_3 generation. Then, BC_3F_3 were tested for their resistance, performances and yield in pea production area of the Royal Project Foundation.

3.5 Morphological characteristics, yield quality and powdery mildew resistance evaluation

3.5.1 Traits character and powdery mildew resistant test

The BC_3F_3 pea hybrid derived from four crosses, No.3 × P309, P309 × No.4, No.5 × P309 and Fang No.7 × P309 as well as parental plants were tested for their resistance and yield performance at two pea production areas of the Royal Project Foundation Station, Khun Wang Royal Project Development Centre, Mae Wang district, and Ang Khang Royal Agricultural Station, Fang district, Chiang Mai. These two locations are located more than 1,000 meters above the sea level. The experiment was conducted using RCBD with 3 replications. The distance between plants and rows were 10 × 10 cm. Plant growth, *i.e.* height, number of nodes and branches, pod width and length, number of seed per pod and number pod per plant,

were recorded. Furthermore, the powdery mildew disease severity on the leaves surface area was scored as described in 3.2. The data were statistic analyzed by SXW version 8.0 computer programs.

3.5.2 Consumer preference trial

Four improved cultivars, BC_3F_3 progenies derived from No.3 × P309, P309 × No.4, No.5 × P309, Fang No.7 × P309, and lines No.3. No.4, No.5 and cultivar Fang No.7, were employed in this study. Pod of improved lines were tested in order to evaluate the preference or perception of consumers. The tester group was the Royal Project shop buyers. Most of them always buy fresh produces as household consumption. A questionnaire was used to investigate the consumer satisfaction on pod characteristics and taste of new pea cultivars compared to their parents. The 5-points hedonic scale was used to measure product acceptance and preference and the scale categories corresponded to 5=like extremely, 4=like very much, 3= like moderately, 2= like slightly and 1= neither like nor dislike. These values were assigned to the attribute of consumer desirable trait, pod size and shape, crispness or toughness, sweetness, colors and scent (Table 3.5). Pods were boiled for 30 min. Random panel tested the pod and wrote down the preference on the questionnaire.

The questionnaire was coded to facilitate data entry. The data were analyzed following Likert scale method (Srisa-ard, 2002) using by by SXW version 8.0 computer programs. The percentages of responses of the attitudinal data were calculated by mean.

Table 3.5 Example of questionnaire done by consumers

Pod characteristic	dislike	Like slightly	Like moderately	Like very much	Like extremely
Size and shape	1	2	3	4	5
Crispness/toughness	1	2	3	4	5
Sweetness	1	2	3	4	5
Color	1	2	3	4	5
Scent	1	2	3	4	5