MOLECULAR BREEDING FOR IMPROVEMENT OF FOLIAR FUNGAL DISEASE RESISTANCE AND OIL QUALITY IN GROUNDNUT (*Arachis hypogaea* L.)

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M.Sc. (Agri.)

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THESIS SUBMITTED TO THE PROFESSOR JAYASHANKAR TELANGANA STATEAGRICULTURAL UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF

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2019

DECLARATION

I, **DNYANESHWAR B. DESHMUKH**, hereby declare that the thesis entitled **"MOLECULAR BREEDING FOR IMPROVEMENT OF FOLIAR FUNGAL DISEASE RESISTANCE AND OIL QUALITY IN GROUNDNUT (*Arachis hypogaea* L.)"** submitted to the **Professor Jayashankar Telangana State Agricultural University** for the degree of **Doctor of Philosophy in Agriculture** is the result of original research work done by me. I also declare that no material contained in the thesis has been published earlier in any manner.

Place: Hyderabad

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Date : 17/05/2019

CERTIFICATE

Mr. DNYANESHWAR B. DESHMUKH has satisfactorily prosecuted the course of research and that thesis entitled **"MOLECULAR BREEDING FOR IMPROVEMENT OF FOLIAR FUNGAL DISEASE RESISTANCE AND OIL QUALITY IN GROUNDNUT (*Arachis hypogaea* L.)"** submitted, is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that neither the thesis nor its part thereof has been previously submitted by him for a degree of any University.

Date: 17/05/2019

(Dr. M. BALRAM)
Chairperson

CERTIFICATE

This is to certify that the thesis entitled **"MOLECULAR BREEDING FOR IMPROVEMENT OF FOLIAR FUNGAL DISEASE RESISTANCE AND OIL QUALITY IN GROUNDNUT (*Arachis hypogaea* L.)"** submitted in partial fulfillment of the requirements for the degree of '**Doctor of Philosophy in Agriculture**' of the Professor Jayashankar Telangana State Agricultural University, Hyderabad a record of the bonafide original research work carried out by **Mr. DNYANESHWAR B. DESHMUKH** under our guidance and supervision.

No part of the thesis has been submitted by the student for any other degree or diploma. The published part and all assistance and help received during the course of the investigations have been duly acknowledged by the author of the thesis.

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Rajendranagar
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DNYANESHWAR B. DESHMUKH

LIST OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

LIST OF APPENDICES

LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Per cent
=	:	Equal to
>	:	Greater than
≤	:	Less than or equal to
°C	:	Degree Celsius
µl	:	Micro liter
bp	:	Base pair
cM	:	Centimorgan
cm	:	Centimeter
CTAB	:	Cetyl Trimethyl Ammonium Bromide
CV	:	Coefficient of Variation
DAI	:	Days After Inoculation
DAS	:	Days After Sowing
dNTP	:	Deoxyribose Nucleoside Tri-Phosphate
EDTA	:	Ethylene Diamine Tetra Acetic Acid
EST	:	Expressed Sequence Tags
et al.	:	and others
FAs	:	Fatty Acids
FAD	:	Fatty Acid Desaturase
FYM	:	Farm Yard Manure
g	:	Gram
HOA	:	High Oleic acid
ICRISAT	:	International Crops Research Institute for the Semi-Arid Tropics
ICGV	:	ICRISAT Groundnut Variety
i.e.	:	That is
ILs	:	Introgression Lines
Indel	:	Insertion or Deletion
K	:	Potassium
KASP	:	Kompetitive Allele Specific Polymerase Chain Reaction
Kg	:	Kilogram
LG	:	Linkage Group
LLS	:	Late Leaf Spot

LSD	:	Least Significant Difference
m^2	:	Meter square
MAS	:	Marker-Assisted Selection
MABC	:	Marker-Assisted Backcrossing
M ha	:	Million Hectares
min	:	Minute
mm	:	Millimeter
MT	:	Million Tons
MUFA	:	Mono-Unsaturated Fatty Acid
N	:	Nitrogen
ng	:	Nano gram
nm	:	Nanometer
no.	:	Number
O/L ratio	:	Oleic acid to Linoleic acid ratio
P	:	Phosphorous
PCR	:	Polymerase Chain Reaction
PUFA	:	Poly-Unsaturated Fatty Acid
PV	:	Phenotypic Variance
QTLs	:	Quantitative Trait Loci
QTL -seq	:	QTL –sequencing
s	:	Sec
SD	:	Standard Deviation
SFA	:	Saturated Fatty Acids
SNP	:	Single Nucleotide Polymorphism
SSRs	:	Simple Sequence repeats
SV	:	Stability Value
Tm	:	Melting temperature
UFA	:	Unsaturated Fatty Acids
viz.	:	Namely
µm	:	Micrometer

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ABSTRACT

Kadiri 6 (K 6) is a popular high yielding, short duration groundnut variety possessing high oil and attractive kernel features, nevertheless K 6 is highly susceptible to late leaf spot (LLS) and rust diseases of groundnut. The present study sought to introgress LLS, rust resistance and high oleic acid traits from donor lines ICGVs 13193 and 15033 respectively into K 6 cultivar. Genotyping for target alleles was performed in first generation with gel based linked SSR markers and in later generations with high throughput Kompetitive allele specific PCR (KASP) based SNPs assay. Backcrossing and selfing resulted in the development 343 BC_1F_2s of which 16 plants were identified with homozygous alleles for rust, LLS and high oleic traits. Evaluation of these backcross progenies resulted in four progenies (BC_1F_2s-76, 216, 225, and 311) resistant to LLS and three progenies (BC_1F_2s-4, 186 and 327) resistant to rust. Morpho-agronomic and nutritional quality analysis revealed three progenies (BC_1F_2s-76, 278 and 296) were superior for morpho-agronomic and nutritional traits over K 6. The pod features *viz*., pod beak, pod reticulation, pod constriction, and pod ridges were attractive over K 6. The higher oil content (>50.0%) was observed in 11 progenies (BC_1F_2s-5, 76, 120, 144, 186, 214, 225, 249, 278, 296 and 327) in comparison to K 6 (49.5%). The oleic acid was higher in three progenies BC_1F_2-76 (81.32%), BC_1F_2-278 (82.8%) and BC_1F_2-296 (83.4%).

These three progenies showed resistance to LLS a disease score of 3.0- 3.5 and rust resistance with a disease score of 4.0. The selected progenies featured with slight pod constriction and pod reticulation. Pod beak was slight in two progenies (BC_1F_2s-76 and 296) and medium in BC_1F_2-278. The pod ridges were absent in BC_1F_2s- 278 and 296 and slight in BC_1F_2-76. All the selected segregants showed higher oil content (>50.0%), oleic acid (>80.0%) and reduced linoleic acid (<10.0%). Advanced breeding lines derived from K 6 for LLS, rust resistance and high oleic traits will be suitable replacement for K 6 especially in disease hot spot locations.

INTRODUCTION

Chapter I

INTRODUCTION

Groundnut or peanut (*Arachis hypogaea* L.) is a high value, nutrient-rich leguminous oilseed, food, fodder and feed crop cultivated in about 114 countries of the world. Majority of groundnut cultivation in the developing countries is done by small and marginal farmers under low-input conditions (Bhauso *et al*., 2014 and Sarkar *et al*., 2014). Groundnut is regarded as the "King of Oilseed Crops" on account of its diversified uses for food, feed, fodder, and industrial purposes (Sahdev, 2015). The seed consists of oil (44-56%), easily digestible protein (22-30%) and vitamins (E, K, and B complex), minerals (Ca, P, Mg, Zn, and Fe), and fiber. Groundnut offers many health benefits like weight gain control (Alper and Mattes, 2002), prevention of cardiovascular diseases, protection against Alzheimer's disease and cancer inhibition (Awad *et al*., 2000).

Globally, out of the total groundnut production, 49% goes for extraction of oil, 41% for food use, and 10% for feed and seed (Singh and Nigam, 2016). Asia is the global leader with a share of 50% and 60% of the world's groundnut area and production, respectively. India ranks first in acreage, occupying 5.80 M ha under cultivation and second in production (6.87 M tonnes), after China (16.68 M tonnes). Groundnut was the third largest exported raw agricultural commodity from India in 2017-18 after rice and cotton (APEDA, 2018). The major states in India producing groundnut include Gujarat (2.33 M tonnes), Tamil Nadu (0.89 M tonnes), Andhra Pradesh (0.80 M tonnes), Karnataka (0.39 M tonnes), Maharashtra (0.33 M tonnes) and Telangana (0.11 M tonnes) (Annual Report, AICRP-G, 2016-17).

Groundnut seed has diverse commercial and industrial uses which are primarily determined by the oil content and the fatty acid composition of the kernels, the latter playing an important role in enhancing shelf life and health benefits.

Mono-Unsaturated Fatty Acid (MUFA), oleic acid (36-67%) and Poly-Unsaturated Fatty Acids (PUFA), linoleic acid (15-43%) comprise 80% of total fatty acids in groundnut (Moore and Knauft, 1989).

The remaining 20% includes Saturated Fatty Acids (SFA) with palmitic acid (~10%), and stearic, arachidic, gadoleic, behenic, and lignoceric acids together making up the remaining 10% (Kavera *et al*., 2014). Linoleic acid and its acyl residues are vulnerable to oxidation, which adversely impacts on oil stability and increases off flavors commonly associated with rancidity of oil (Patel *et al*., 2004). A higher level of palmitic acid is associated with cardiovascular disease (CVD) (Hu *et al*., 2001).

Groundnut and groundnut-based products with the high-oleic content have a shelf life five to 10 times longer than that of normal groundnut. Its neutral flavor and odor make the oil highly suitable for a wide range of food-related uses. Consumption of high oleic (with >80% oleic acid) groundnuts promotes a ratio of healthier High-Density Lipoprotein (HDL) to Low-Density Lipoprotein (LDL), lowers CVD risk by 15% (Wang *et al*., 2017) and reduces triacylglycerol and blood glucose levels (Vassiliou *et al*., 2009). Groundnut oils with >80% oleic acid content are comparable to olive oil which contains about 75% oleic acid. Oils with a higher proportion of mono-unsaturated fatty acids are amenable to heat at high temperatures without smoking, leading to faster cooking time and absorption of less quantity of oil in fried food (Sarvamangala *et al*., 2011).

High oleic groundnut is preferred by food industry for shelf life benefits and meets the demand of the growing Asian market of groundnut-based products like chocolate bars, groundnut butter, groundnut cookies, roasted groundnut etc. Thus, multinational confectionary companies based in Asia are sourcing tons of high oleic groundnuts from Australia and the United States. However, cross-country procurement and import of groundnut increases the input cost for processing industries.

Hence, leading food companies seek opportunities to locally source high oleic groundnuts from India and other countries in Asia and Africa where they operate. The availability of high oleic groundnut varieties also improves the income of smallholder groundnut farmers as industries pay a premium price for high oleic groundnut.

For altering the fatty acid composition, concentrated effort has been made in vegetable oil breeding programs for the identification and generation of high oleic lines in different oilseed crops including groundnut (Janila *et al*., 2016a and Bera *et al*., 2018), soybean (Haun *et al*., 2014), and sunflower (Martinez-Rivas, 2001).

The foundation in high oleic groundnut breeding was laid by Norden *et al*. (1987) who identified the natural HO mutant lines F435-2-1 (79.91%) and 435-2-2 (79.71%) while screening of a large number of genotypes at Gainesville and Marianna locations, respectively. Subsequently, F 435 derived high oleic groundnut variety 'SunOleic 95R' was released in the USA (Gorbet and Knauft, 1997). Later on, Chu *et al*. (2011) developed 'Tifguard High O/L' by pyramiding high oleic trait with nematode resistance by deploying Marker-Assisted Selection (MAS). A cultivar, 'TifNV-High O/L' was developed with resistance to the peanut root-knot nematode and spotted wilt, with high oleic acid (Holbrook *et al*., 2017). So far, more than 90 high oleic groundnut genotypes have been developed in the United States of America, China, Brazil, Korea, South Africa, Australia and Argentina using conventional breeding approaches (Wang *et al*., 2015, Janila *et al*., 2016a, Bera *et al.,* 2018 and Nawade *et al*., 2018). Some groundnut lines GJG 16, GJG 20, GJG 21, GJG 22, TMV 10, Kadiri 71-1 (Nawade *et al*., 2016); GM 4-3 and GM 6-1 (Nadaf *et al*., 2017) were developed in India with an elevated oleic acid content of ca. 65%. However, they don't meet the requirement of the food industry that requires a high oleic acid content of ca.80%. Recently at the AICRP-G workshop held in April 2018, guidelines were released for high oleic groundnut varieties that suggests a high oleic groundnut should have an oleic acid content of 80±2% when measured on fully mature kernels. These guidelines will pave the way to commercialize high oleic groundnut varieties in India.

Fungal diseases like leaf spots, rust, root rot, stem rot, pod rot, and blight causes severe pod yield penalty and quality loss of both pods and fodder. Among the foliar fungal diseases, rust caused by *Puccinia arachidis* Speg, early leaf spot by *Cercospora arachidicola* [Hori] and Late Leaf Spot (LLS) by *Phaeoisariopsis personata* [(Berk. and Curt) Deighton] are widely distributed, most destructive and economically important in India. LLS and rust can cause 50-70% pod yield loss (Subrahmanyam *et al.*, 1984 and Janila *et al.,* 2016b) under favourable conditions.

The cultivation of groundnut varieties resistant to these foliar fungal diseases is one of the practically feasible, economic and environmentally sustainable options. Host-resistance to LLS and rust have been reported in cultivated and wild species of *Arachis* spp. (Subrahmanyam *et al*., 1983., Pande and Rao, 2001 and Sudini *et al*., 2015).

The level of LLS and rust resistance in wild species was higher and interspecific derivatives such as ICGV 86855 (CS 16), ICGV 86687 (CS 16 –B2 –B2) (ICRISAT Annual Report, 1987) and VG 9514 (Varman, 1999) were developed for use in breeding program to introgress resistance from wild species.

Recently, the entire genome of groundnut and its diploid progenitors *A. duranensis* and *A. ipaensis* has been sequenced and analyzed (Bertioli *et al*., 2015 and Chen *et al*., 2016). This laid the foundation for understanding the genetics of traits in groundnut. Markers are available for the high oleic trait, and resistance to rust and LLS. The QTL region on linkage group (LG) A03 explains up to 82.62% of the phenotypic variance (PV) for rust resistance and a major QTL on LG A02 explains up to 62.34% PV for LLS resistance. One region present on LG A03 represents three QTLs each for LLS (up to 67.98% PV) and rust (up to 82.96% PV) (Sujay *et al*., 2012 and Pandey *et al*., 2017). The use of these validated QTLs/genes for rust and LLS resistance holds promise for groundnut improvement through marker-assisted breeding.

Kadiri 6 (K 6) is a popular groundnut variety among the farmers well-known for its high yield and stability. It was developed at Kadiri Research Station, Acharya N. G. Ranga Agricultural University (ANGRAU) of Andhra Pradesh. It was released in 2005 for cultivation in the Indian states of Andhra Pradesh (AP) and Telangana.

K 6 is cultivated widely in AP and Telangana, and preferred by farmers and traders for high yield potential and desirable plant, pod and seed characteristics. However, it is highly susceptible to LLS and rust that reduces the pod yield and also fodder quality and yield. With the availability of molecular markers for LLS and rust (Khadikar *et al*., 2010., Sujay *et al*., 2012 and Pandey *et al*., 2017) and high oleic acid content (Chu *et al*., 2009 and Chen *et al*., 2010) there is ample scope to improve this mega variety for these three traits. Development of groundnut varieties with pyramided traits is a key strategy to meet the needs of the production ecologies as well as the needs of consumers and industries.

Plant breeding coupled with genomic resources and tools like molecular markers would enhance the precision and speed of developing groundnut cultivars with resistance to LLS, rust and improved nutritional quality. The work reported here, attempts to improve K 6 for LLS and rust resistance and oil quality especially oleic acid content using marker-assisted backcrossing (MABC) approach.

The objectives of the current investigation were framed as follows:

1) Introgression of rust and late leaf spot (LLS) resistance QTLs into K 6 cultivar
2) Introgression of alleles for high oleic acid into K 6 cultivar
3) Combining QTLs for rust, LLS resistance and high oleic acid alleles in the background of K 6 cultivar by adopting marker-assisted backcrossing (MABC)
4) Phenotypic evaluation of backcrossed lines for rust and late leaf spot resistance
5) Evaluation of backcrossed lines for fatty acids and morpho-agronomic traits

REVIEW OF LITERATURE

Chapter II
REVIEW OF LITERATURE

Groundnut improvement for late leaf spot (LLS), rust resistance and high oleic acid traits entails hybridization, utilization of molecular markers linked to these traits and evaluation of backcross lines under field conditions. The success of such cultivar improvement program depends on thorough understanding of biochemical pathways, sources of resistance, genetics of resistance, breeding methods, components of disease resistance, screening methods available for disease resistance, etc. In this chapter, the literature on the following aspects is reviewed.

2.1 Groundnut and its importance

2.2 Foliar diseases of groundnut: Late leaf spot and rust

2.3 Host-plant resistance

2.4 Late leaf spot and rust resistance: physiological and biochemical insights

2.5 Genetics studies and mapping for late leaf spot and rust resistance

2.6 Progress in breeding for late leaf spot and rust resistance

2.7 Oil quality parameters in groundnut

2.8 Morphological evaluation of groundnut

2.1 Groundnut and its importance

Groundnut or peanut (*Arachis hypogaea* L.) is an important oilseed, food and feed crop grown worldwide in 118 countries. Groundnut is cultivated in an area of 27.94 million hectares (Mha) with a production of 47.09 million tons (MT) of pods in 2017. India ranks first in an area occupying 5.30 Mha under groundnut cultivation, and second in production with 9.17 MT of pod yield, after China (17.15 MT) (FAOSTAT, 2017). India and China are the largest producers of groundnut and have a high consumption in the form of confectionery and as cooking oil.

Besides catering to the domestic market demand, India also exports groundnut derived processed products, hand-picked selected groundnuts and oil cake forms. Although uses of groundnut in various countries differ greatly, a total of more than 50% of groundnut produce is crushed for oil extraction. Consumption of edible vegetable oils at the global scale has drastically increased from 87.8 MT in the year 2000 to 186.5 MT in 2016 (Nawade *et al*., 2018).

The conventional use of groundnut is being extended for making products *viz*., butter, confections, chocolates, candies, boiled, salted, roasted nuts, fried and coated with chickpea flour etc. Groundnut based ready-to-use therapeutic food (RUTF) has shown to be effective in the rehabilitation of severely malnourished children and facilitates home-based therapy for them (Manary, 2006). Groundnut seed contains oil (40-56%), protein (20-30%), carbohydrate (10-20%), and several nutritional components such as vitamin E, niacin, calcium, magnesium, phosphorus, zinc, iron, riboflavin, thiamine, and potassium (Dean *et al.*, 2009). Groundnut oil is rich in calorific value (884 calories/ 100 g of oil) and a good source of plant sterol especially β-sitosterol. β-sitosterol is known to have anticancer properties and reduces cholesterol level up to 10–15% by inhibiting the cholesterol absorption (Awad and Fink, 2000). Groundnut oil is also a source of valuable antioxidants vitamin E, resveratrol, phenolics and flavonoids which are good for human health to neutralize the harmful free radicals, stresses and maintains cell membrane integrity (Meredith and Alfred, 2003). The groundnut seed with high oil (≥50%) and high oleic acid (75–80%) contents can be an excellent alternative to canola and olive oil, which are exorbitant by 2.5 and 11 times, respectively. Currently, countries like Australia, Argentina, United States of America, Brazil, South Africa, China and Israel are producing high oleic groundnuts on a large scale (Barkley *et al.*, 2013) but such trend is likely to be followed in other parts of the world.

In the Asian and African countries, a shortage of good quality fodder for the livestock is a serious concern. Hence the demand for fodder is fulfilled through the use of left over residues of crops either as raw or for silage preparation. Indeed, groundnut haulms are more palatable and rich in protein compared to stovers of cereals which have low N, high fiber content, and poor digestibility (Frimpong *et al*., 2017). In India, groundnut haulms contribute more than 30% to the fodder from leguminous residues (Nigam and Blummel, 2010).

Hence, groundnut not only benefits the farmers through pod yield but also though the haulms which are utilized as fodder for livestock.

Groundnut fixes atmospheric nitrogen and adds about 80 kg of nitrogen (N) per hectare per season (Herridge *et al*., 2008). The groundnut residues can contain >160 kg N ha^{-1}, are less lignified (~5% lignin), and are rich in N, even if the crop is harvested while green (Mafongoya *et al*., 2006). Hence, groundnut is suggested either as an intercrop or in crop rotation with cereals to maintain the fertility of the soil and helps in reducing soil erosion.

A variety of industrial products for domestic use or exportation such as paint, varnish, lubricating oil, leather dressings, soaps, candles, cosmetics, some textile fibers, insecticides, and nitroglycerine are derived from groundnut oil (Singh and Diwakar, 1993). The shells of groundnut are used in the manufacture of plastic, wallboard, abrasives, fuel and cellulose (Janila *et al*., 2013b). The groundnut shell based biopolymeric materials was found to be a good material for adsorption of heavy metal ions like Cu(II), Ni(II) and Zn(II) from their aqueous solutions (Shukla and Pai, 2005).

2.2 Foliar diseases of groundnut: Late leaf spot and rust

Among biotic constraints that limit groundnut yield, foliar fungal diseases are the most lethal. The foliar fungal diseases, leaf spot and rust, are commonly present wherever the groundnut is cultivated, but their incidence and severity vary between locations and seasons. These diseases are prevalent during the rainy season in groundnut growing area of Indian states exhaustive in South India where the dry and humid condition favors the development and spread of the disease (Subrahmanyam and McDonald, 1983).

An estimated global yield loss of US$ 600 million annually due to LLS alone has been reported (Janila *et al*., 2016a). Each disease alone is capable of causing substantial yield loss but when they occur together, losses are further increased.

In India, LLS and rust normally occur together and can cause yield losses up to 70% (Subrahmanyam *et al.,* 1980 and Singh *et al.*, 2011); and reduce digestibility of haulms by 22% (Pande *et al*., 2003). These diseases also have an adverse influence on the recovery of pods, quality of seeds and haulms.

Cercospora or tikka leaf spots (early and LLS) are the most important fungal diseases of groundnut. LLS is caused by *Phaeoisariopsis personata* (Berk. and M.A. Curtis) van Arx., and prevalent during the rainy season in many groundnut-growing areas of the world. The infestation of disease starts around mid-August when the fungal spores germinate and penetrate the leaves through stomata located on the abaxial surface.

Round-shaped black lesions become visible within one week of fungal germination. Since the spores are deposited in the soil, LLS disease usually starts from the bottom layer of the plant canopy and moves upward. As the fungal lesions enlarge, coalescence of lesions often is observed in highly susceptible lines (Subrahmanyam *et al*., 1995). Sporulation occurs in 20 to 30 days after infection. Secondary infection from freshly produced spores is common in the groundnut crop with long maturity period (>140 days). Predominantly, leaf spot reduces the yield by reducing the available photosynthetic area, localized damage of leaf tissues and defoliation. Highly susceptible genotypes can lose all their leaves one month prior to maturity (Pande and Rao, 2001).

Rust caused by *Puccinia arachidis* (Spegazzini) is an important foliar fungal disease of groundnut in almost all groundnut growing areas of the world (Subrahmanyam *et al*., 1995). Groundnut rust appears in the field under warm and humid conditions and spreads quickly by repeated infection cycle of uredospores. The appearance of symptoms of rust starts 8-10 days after infection with the occurrence of whitish flecks on the abaxial surface.

A day later, yellowish flecks appear on the upper leaf surface and orange, red/brown-colored pustules (uredinia) begin to form on the lower surface of the leaves. The pustules rupture after about 2 days of appearance to expose circular or oval uredospores which are dark orange at first but become cinnamon brown with maturity (Savary *et al*., 1989). These spots can rapidly increase in size during periods of wet and warm weather. Epidemic development is favoured by continuous warm temperatures (>22° C) with wet weather or high humidity (>78%).

In the absence of chemical control measures, the severe incidence of disease can completely destroy the crop. Depending on genotype, time and stage of occurrence, rust infestation causes about 50-70% of pod and fodder yield losses and also adversely effects the quality of produce (Subrahmanyam *et al*., 1984).

These enormous losses in the groundnut yield and fodder can be minimized either with the use of plant protection chemicals and fungicides or cultivation of resistant cultivars (Pande *et al.*, 2003). The preventative schedule spray of fungicides to control LLS and rust is common practice among groundnut growers of developed countries where mechanical resources are good and input cost goes much higher. But, the extensive use of fungicides leads to a residual effect on foliage and creates environmental pollution.

Hence, the use of resistant cultivars becomes economically feasible and environmentally sound approach. The adoption of resistant cultivars minimizes losses at the farm level reduces the input cost and maintains good product quality which in turn increases the profitability of the farmers. For effective management of LLS and rust diseases in groundnut, breeding work should be focused on utilizing the resistance genes/QTLs available in the gene pool. Host resistance is one of the strategies that have been exploited in food crops to combat many diseases such as bacterial leaf blight and blast diseases in rice (Sundaram *et al*., 2008 and Prashanthi *et al*., 2017), stem rust in wheat (McIntosh *et al*., 1995), stem rust and spot blotch in barley (Steffenson and Smith, 2006), potato late blight (Rodewald and Trognitz, 2013), anthracnose in sorghum (Mengistu *et al*., 2018).

2.3 Host-plant resistance

Understanding the plant-pathogen interaction mechanism and components of the plant defense mechanism by which plants resist infection or become susceptible to a microbial pathogen are very important to breed resistant cultivars.

Based on the screening in field and laboratory conditions, Subrahmanyam *et al*. (1983) and, Pande and Rao (2001) identified that *A. batizocoi* (PI 298639, PI 338312), *A. duranensis* (PI 219823), *A. cardenasii* (PI 262141), *A. chacoense* (PI 276235), *A. pusilla* (PI 338449), *A. villosa* (PI 210554), *A. correntina* (PI 331194), and *A. kuhlmannii* (ICG 8954) are immune to rust. However, introgression of disease resistance alleles from wild diploids to allotetraploid groundnut is hindered by sterility barriers, mostly due to genomic and ploidy differences between *A. hypogaea* and the related species. In spite of this limitation, significant progress has been made to transfer the resistant alleles from wild species to *A. hypogaea* especially from *A. cardenasii*, a wild A-genome diploid relative to cultivated groundnut.

Leaf spot resistant line, CS 16 derived from interspecific hybridization (*A. hypogaea* × *A. cardenasii*) and screened in India led to identify two LLS resistant lines *viz*., ICGV 86855 (CS 16) and ICGV 86687 (CS 16 –B2 –B2) was identified (ICRISAT Annual Report, 1987). Later on, CS16 was used as a donor source to breed LLS and rust resistant cultivars.

One such cultivar, GPBD 4 derived by crossing KRG 1 and ICGV 86855, is a very popular variety released in India and is used as resistant check in India for LLS and rust scoring (Gowda *et al*., 2002), and also as donor parent for LLS and rust resistance (Varshney *et al*., 2014). Also, varieties such as York, Georgia-07W, Georgia-03L, Southern Runner, Florida MDR98 and Georgia Green with improved leaf spot resistance were bred and are commercially grown in Southeastern part of USA (Culbreath *et al.,* 2009).

Sudini *et al*. (2015) screened mini-core collection for two years and identified eleven accessions (ICGs 11426, 11088, 6022, 12625, 15419, 4389, 111, 4343, 4527, 4746, and 6993) resistant to rust. Two accessions (ICG 11426 and ICG 12625) were resistant to LLS. The selected groundnut accessions (all are *A. hypogaea*) ICGs 4389 (origin: India), 6993 (origin: Brazil), 11426 (origin: India), 6022 (origin: Sudan), 4746 (origin: Israel) and 11088 (origin: Peru) showed superior reactions in terms of resistance, lower rates of LLS and rust disease progress and also good pod yields.

The resistance source against LLS and rust was reported to be present in primary, secondary, and tertiary gene pool of *Arachis*. But, only primary and secondary gene pools are considered as valuable sources of resistance due to their cross-compatibility with the cultivated *Arachis* species. The LLS resistance has been identified in wild diploids such as *A. cardenasii* (Company *et al*., 1982), *A. stenosperma* (Leal-Bertioli *et al*., 2009), *A. diogoi* (Kumar and Kirti, 2015) and *A. batizocoi* (Zhou *et al*., 2016).

Apart from the challenge of identifying and utilizing the resistance sources in groundnut, the genetic nature of the resistance to foliar diseases also limits the progress in resistance breeding. Leaf spots and rust occur together and interfere with each other making the selection inefficient (Sukruth *et al*., 2015). Also, the tight linkage between disease resistance and, undesirable pod and kernel features demands raising large segregating population and precision in screening and selection.

2.4 Late leaf spot and rust resistance: physiological and biochemical insights

In field condition, groundnut plants cope with the biotic stresses by a combination of physiological, and biochemical responses controlled by many genes (Kumar and Kirti, 2015). LLA and rust affect the growth, cell cycle, and metabolism of groundnut encumbering the genetic potential of the cultivar.

These stresses reflect in visible traits like lesions of *P. personata* on leaves, branches, stem, and pustules of rust, defoliation, stunted growth and premature death of plant and take a toll on pod yield and haulm quality. The series of physiological and biochemical responses that have been reported in groundnut against LLS and rust have been reviewed hereunder.

2.4.1. Late leaf spot

Resistance to LLS in *A. hypogaea* is due to longer latent period, reduced sporulation, and less defoliation (Nevill, 1981). Mahapatra (1982) reported that leaves of groundnut infected by *C. personata* contained high quantity of reducing sugars than the healthy ones. The sharp decline in non-reducing sugars, immediately after infection, while an increase in the total sugars was observed as the disease progressed.

In the glasshouse screening of groundnut against LLS, the lesion diameter, defoliation, and sporulation were found to be correlated with field disease score (Subrahmanyam *et al.,* 1983). Brahmachari and Kolte (1983) studied the leaf anatomy of groundnut cultivars resistant to *C. arachidicola* and *Cercosporidium personata*. They found fewer stomata/unit leaf area and a thicker palisade layer in resistant cultivars than susceptible cultivars.

At all stages of plant growth resistant plants had more total chlorophyll and higher total phenol content. Sporulation, lesion size, and latent period are the important components of resistance to LLS and are highly correlated with each other and with the percentage of leaf necrotic area (Chiteka *et al.,* 1988).

Bera *et al.* (1999) found that *Cercospora* resistant groundnut genotypes were capable of maintaining a higher level of chlorophyll, soluble sugars, protein and phenol contents. Selection of varieties with an increased amount of phenols in the leaves was suggested for development of tikka disease resistant genotypes.

Motagi (2001) reported that the incubation period, lesion size and lesion on main-stem as the most important components of resistance and having a strong association with field disease score, defoliation and remaining green leaf area.

Dwivedi *et al*. (2002) observed longer incubation period, longer latent period, lesser lesions per leaf, smaller lesion diameter, lower sporulation index, lesser percentage leaf area damaged, and lower disease scores in the resistant genotype as compared to susceptible genotype and also reported high correlation among components of resistance.

According to Pensuk *et al*. (2003), the genotype NC 17135 exhibited high resistance to LLS because it had lesser lesion number per 100 cm^2 of leaf area and least sporulation compared to all the commercial cultivars, NC 9, Tainan 9 and Lampang that were highly susceptible.

Jyosthna *et al*. (2004) reported higher total phenols, peroxidase and polyphenol oxidase in LLS resistant cultivar, FDRS-10 and upon infection, increased further, in all cultivars under study. Significant differences in banding profiles of phosphatase and esterase isozymes were observed in all cultivars.

2.4.2. Rust

Resistance to rust in A. *hypogaea* is attributed to the prolonged incubation period, low infection frequency, less number of pustules, smaller pustules, and less ruptured pustules and leaf area damage less sporulation and inefficient germinability of spores produced in the uredia (Subrahmanyam *et al.,* 1983).

Infection frequency, pustule diameter, percent ruptured pustules, and leaf area damage is correlated with each other and with field rust score. Velazhahan and Vidhyasekaran (1994) studied the alteration in phenolics contents of groundnut leaves infected by *P. arachidis* (rust) and revealed that there were higher phenol and ortho-dihydroxy phenol contents in resistant varieties as compared with susceptible varieties. These contents also increased after infection in both susceptible and resistant varieties.

Rao *et al.* (1996) explored the electron microscopy for studying rust resistant groundnut genotypes and found that in susceptible genotype, TS 32-1, infection pegs developed from appressoria over stomata and enter the substomatal cavity through the openings between guard cells.

While in the resistant genotype, PI 259747, the infection peg either failed to enter through the stomata or the infection structure disintegrated in the substomatal cavity. It was also suggested that phytoalexins may be involved in disease resistance. They found that the rate of phytoalexin accumulation was faster in the resistant genotype.

Sankara *et al*. (1996) investigated the accumulation of phytoalexins in a rust-susceptible (KH 241-D) and a rust-resistant (PI 259747) genotype of groundnut They observed that the total phytoalexin concentrations in the resistant genotype at 2, 6, 15 and 25 days after inoculation were always higher than those of the susceptible genotype.

The chitosan-treated leaves had elevation in endogenous salicylic acid, intercellular chitinase, and glucanase activity leading to reduced germination of uredospores, reduced number of lesions, lesser lesion diameter, and fewer sporulation of *P. arachidis* (Sathiyabama and Balasubramanian, 1998).

Sunkad and Kulkarni (2006) in their study characterized resistant (GPBD-4 and DH-22), moderately resistant genotypes (K-134 and R-8808) with higher cuticular and epidermal cell thickness with lesser epidermal cells, size (length, breadth) and a number of stomata and more wax content at later stages of crop growth than susceptible genotypes (KRG-1 and TMV-2). The sugars, phenol, ortho-dihydroxy phenol and protein contents were more in resistant genotypes than susceptible (KRG-1 and TMV-2) genotypes.

Sudhagar *et al*. (2009) studied the alterations in the activity of peroxidase (PO), polyphenol oxidase (PPO), ascorbic acid oxidase (AAO) and chitinase at 80 and 90 days after sowing (DAS) both in susceptible and resistant segregants along with parents after rust infection in groundnut. Both susceptible and resistant segregants manifest increased activity of all of these three enzymes, the magnitude was higher in resistant segregants. The activity of ascorbic acid oxidase and chitinase were high at 90 DAS, while polyphenol oxidase and peroxidase exhibited maximum activity at 80 DAS. Four additional isoforms of PO, PPO and prominent expression of a 56 kDa protein were observed in resistant genotypes.

2.5 Genetic studies and mapping for late leaf spot and rust resistance

With advances in technologies, the genomic approaches are being used in plant breeding to enhance selection precision and reduce breeding cycles. Genomic tools help to decipher the location and function of several stress-responsive genes. In the context of groundnut leaf spot and rust, genomics tools have paved a long path starting from simple QTL mapping techniques to next-generation high throughput genomics. Identification of genomic regions/QTLs conferring resistance would help breeders to develop more efficient and effective breeding stocks through marker-assisted selection (MAS).

Molecular markers are being widely used for identification, screening, and selection of LLS and rust resistance genes/ QTLs in groundnut cultivars. They evolved as a tool of choice when it comes to complex trait like LLS and rust. Many studies have identified QTLs for LLS and rust resistance in groundnut.

Luo *et al*. (2005) combined cDNA microarray and real-time PCR to identify the differentially expressed genes that may control resistance to leaf spot disease in two groundnut genotypes *viz*., C34-24 (resistant) and GT-YY20 (susceptible). They identified seventeen genes as putative candidate genes for LLS resistance. In those identified genes, a lipid-transfer protein (LTP) precursor as an antifungal protein showed the differences between the resistant and susceptible cultivars.

In a gene expression profiling study, Nobile *et al*. (2008) identified more than 700 groundnut unique expressed sequence tags (EST) involved in several aspects of the early stages of *C. personatum* pathogenesis. A transcript showing sequence similarity to LTP, *O*-methyltranferase was highly induced in *C. personatum* resistant groundnut genotype cv. 850. They hypothesized LTPs an antifungal protein, plays a role in wax or cutin deposition on the cell walls of expanding epidermal cells and certain secretory tissues. LTPs were found in constructing and reinforcing cellular surfaces transporting HR-induced molecules to systemic response.

Burow *et al.* (2008) identified five RFLP markers for leaf spot resistance in BC_3F_2 lines in greenhouse studies. They identified three QTLs for the incubation period, one each for latency period, and lesion number and diameter. Those QTLs for latency period and lesion number were overlapping, suggesting either linkage between the two or a QTL with pleiotropic effects.

Leal-Bertioli *et al.* (2009) found five QTLs for LLS resistance on an SSR-based map using an F_2 mapping population derived from the cross between *A. duranensis* and *A. stenosperma*. The results suggested additive or partial dominance gene action. One QTL explained almost half of the phenotypic variance observed and some QTLs mapped near resistance gene analogues-based markers.

Mondal and Badigannvar (2010) studied the association of SSR markers with rust and LLS resistance in a set of 20 cultivated groundnut genotypes differing in resistance against both the diseases. The associated five SSR loci explained 25.2-48.6% of phenotypic variation (PV) for rust and four SSR loci with 25.2-42.8% PV for LLS.

The studies on infection mechanism of LLS and rust pathogen on *A. stenosperma* and *A. hypogaea* revealed that the adhesion, germination of spores and hyphal proliferation occurred in both the species. However, the infection was more limited in *A. stenosperma* than in *A. hypogaea* and no successful penetration was observed in the former. These data suggest that in *A. stenosperma*, infection is hampered at the stage of penetration stage (Leal-Bertioli *et al*., 2010).

A total of 268 recombinant inbred lines of a mapping population TAG 24 × GPBD 4 segregating for LLS and rust were used for QTL mapping. Phenotyping coupled with QTL mapping identified 11 QTLs for LLS (explaining 1.70–6.5% PV) in three environments and 12 QTLs for rust (explaining 1.7–55.2% PV). Further, composite interval mapping (CIM) and single marker analysis (SMA) identified a major QTL associated with rust ($QTL_{rust}01$), contributing 6.9–55.2% PVE (Khedikar *et al.*, 2010).

Mondal *et al*. (2012) developed 259 EST-SSR markers by mining 5,184 *Arachis hypogaea* ESTs from NCBI database. They found two EST-SSR markers *viz*., SSR_ GO340445 and SSR_HO115759 closely linked to a rust resistance gene at 1.9 and 3.8 cM distances, respectively in the genetic map of 5,184 *A. hypogaea* ESTs.

Shoba *et al.* (2012) utilized an LLS susceptible genotype (TMV 2) and the LLS resistant genotype (COG 0437) and their F_2 population for marker analysis. The bulked segregant analysis (BSA) using SSR markers *viz*., PM 375162, PGPseq5D5220 and PM 384100 distinguished between the resistant and susceptible bulks.

The phenotypic variation explained by these markers was ranged from 48% for PM 375 to 59% for pPGPseq5D5 and PM 384 which indicated their potential for marker-assisted breeding for LLS over a wide range of genotypes.

Comprehensive QTL analysis detected a total of 28 QTLs for LLS and 15 for rust in two RIL populations, obtained from the cross between TAG 24 × GPBD 4 and TG 26 × GPBD 4. A major QTL for LLS, namely $QTL_{LLS}01$ (GM1573/GM1009-pPGPseq8D09), with 10.27–62.34% phenotypic variance explained (PVE) was detected in six environments. One genomic region presents on linkage group AhXV contained three QTLs each for LLS (up to 67.98% PVE) and rust (up to 82.96% PVE) (Sujay *et al.*, 2012).

Wang *et al.* (2013) identified 37 QTLs possessing 6.61-27.35% PVE for leaf spots in a population from a cross Tifrunner × GT-C20. Based on multi-environment phenotyping data in F_8 and higher generations.

Janila *et al.* (2013b) studied the inheritance of resistance to LLS disease in three crosses and their reciprocals involving two resistant interspecific derivatives ICG 11337, ICG 13919 and a susceptible cultivar JL 24. They reported that resistance to LLS in groundnut is controlled by both nuclear and maternal factors and among factors, additive gene action controlled majority of the variation.

Genetic engineering has opened up the opportunity to exploit the disease resistance genes by transferring them into same or different crop species. Groundnut transformed with a rice chitinase gene (*Rchit*) has shown enhanced resistance to LLS, rust and *Aspergillus flavus* infection (Prasad *et al*., 2013).

A total of 22 SSR markers were linked to rust and LLS resistance in a study carried out on 95 diverse genotypes. The maximum numbers of rust and LLS linked markers were located on the linkage group (LG) 03 (eight SSRs) and 04 (Gajjar *et al*., 2014).

A total of 233 differentially expressed genes involved in cell wall strengthening, hypersensitive cell death and resistance related proteins in *A. diogoi* were identified in gene expression study. Two-dimensional gel electrophoresis (2D-GE) analysis data revealed that the oxygen-evolving enhancer protein, F-box protein, Sedoheptulose-1,7-biphosphatase, Glyceraldehyde- 3-phosphate dehydrogenase, Dihydroflavonol

reductase, Phytochrome A and Photosystem-II chlorophyll a/b binding protein were up-regulated upon pathogen challenge (Kumar and Kirti, 2015).

RIL population was derived from a cross of TAG 24 (LLS and rust disease susceptible cultivar) and GPBD 4 (resistant cultivar). Among the five QTLs mapped on B03 linkage group, three QTLs showed high PVE for LLS and rust, and two QTLs showed high PVE for rust. The QTL flanked by GM2009-IPAHM103 had PVE of 44.5% and 53.7%, for LLS and rust response respectively. Another genomic region on AhXII (B10 linkage group of B genome) contained a QTL flanked by GM1839-GM1009 which had a PVE of 14.1–35.2% for LLS resistance (Kolekar *et al.*, 2016).

A total of 20 QTLs were identified in a RIL population obtained from the cross Zhonghua 5 and ICGV 86699 for LLS, of which two QTLs were identified in multi-environments. Six QTLs with PVE more than 10% was observed (Zhou *et al*., 2016).

The study based on the F_2 generation of 15 crosses for the inheritance of resistance to LLS disease was found to be governed by duplicate recessive genes wherein susceptibility being dominant to resistance (Kumar *et al.*, 2016).

Groundnut plants, transformed with synthetic defensin fusion genes from fenugreek (*Tfgd2*) and radish (*RsAFP2*) linked by a linker peptide, were found to have enhanced resistance to ELS and LLS infection over its wild type (cv. GG 20) (Bala *et al*., 2016).

The RIL mapping population (TAG 24 × GPBD 4) comprising of 266 individuals were used for whole-genome resequencing based approach referred to as 'QTL-seq'. A QTL region on A03 chromosome was identified for LLS (67.98% PVE) and rust (82.62% PVE) resistance. The QTL region on A03 was resolved to 3.06 Mb (131.60–134.66 Mb). Sequence analysis of bulks for rust and LLS with reference-guided resistant parent assembly identified 3136 single-nucleotide polymorphisms (SNPs) for rust and 66 SNPs for LLS (Pandey *et al.*, 2017).

The RIL population derived from the cross Florida-07 × GP-NC WS 16 was used to map QTLs for LLS using 'QTL-seq'. The candidate QTLs were identified on three chromosomes, A05, B03, and B05 with the estimated regions for each QTL being 4.7 Mb, 1.2 Mb and 3.4 Mb respectively. Linear regression of each marker associated with QTLs explained 10%, 5%, and 2% (B05, B03, and A05, respectively) of the variance in four years of data (Clevenger *et al.*, 2018).

A consensus rust QTL was identified within a 1.25 cM map interval of the A03 chromosome in cultivated groundnut. This map interval contains a TIR–NB–LRR R gene and four pathogenesis-related genes. The genetic map of 1.25 cM map interval contained 331.7 kb in the physical map of *A. duranensis* and had a TIR–NB–LRR category R gene (*Aradu*.Z87JB) and four glucan endo-1,3 β glucosidase genes (*Aradu.RKA6 M*, *Aradu.T44NR*, *Aradu.IWV86* and *Aradu*. *VG51Q*) (Mondal and Badigannavar, 2018).

2.6 Progress in breeding for late leaf spot and rust resistance

Breeding for biotic stress resistance has been the key to sustain groundnut production against several biotic stresses. Conventional breeding has established many milestones for improvement of LLS and rust resistance in groundnut.

The elaborate phenotypic screening programs in India and abroad have yielded germplasm lines with resistance to LLS and/or rust. But most of them were Valencia landraces originating from Peru, with a number of undesirable attributes like thick shell, low productivity, poor adaptation, late maturity, highly reticulated and constricted pods which are commercially unacceptable (Subrahmanyam *et al.,* 1995 and Singh *et al.,* 1997). However, later, screening of the germplasm originating from secondary centers of diversity resulted in the identification of resistance sources in good agronomic backgrounds (Singh *et al*., 1997 and Gowda *et al*., 2002). Such genotypes are used as donor material for incorporating disease resistance into local elite cultivars (Gowda *et al.*, 2002 and Janila *et al.*, 2016a).

'Southern Runner' was the first moderate LLS resistant cultivar to be released in the USA (Gorbet *et al*., 1987). At ICRISAT, breeding for economically important foliar fungal diseases has resulted in the development of several genotypes with significant levels of resistance to LLS and rust (Singh *et al*., 2003), which are either released for cultivation in several countries of Asia or have been used as parents in national breeding programmes. Among them LLS resistant cultivars released in India are RG 141, ICG (FDRS) 4, ICGV 86590, ICGV 86325, K 134, Girnar 1, GBPD 4, R 8808, ALR 1, ALR 2, ALR 3, BSR 1, VRI 5 and CSMG 84-1. In the USA, Southern Runner, Florida MDR 98, C-99R, Georgia-01R and Georgia-05E were released as resistant to LLS (Nigam, 2015).

The genotypes *viz.*, ICG (FDRS) 4, ICG (FDRS) 10, ICGV 86590 and GBPD 4 are released in India as rust resistant cultivars (Nigam, 2015). Groundnut improvement programs have relied largely on phenotyping tools that include screening under field and controlled environments to breed LLS and rust resistant varieties (Janila *et al*., 2013b). As compared to the cultivated *A. hypogaea*, very high levels of resistance to LLS and rust were reported in wild species of groundnut (Stalker and Moss, 1987) and these were utilized to derive interspecific derivatives such as ICGV 86855 (CS 16).

The line, ICGV 86855, one of the parents of GPBD 4, is an interspecific derivative between *A. hypogaea* and *A. cardenasii* and shows resistance to both LLS and rust (ICRISAT Annual Report, 1987). However, the use of resistance from wild species in groundnut improvement is limited as a consequence of associated linkage drag resulting in delayed maturity, undesirable pod and kernel features. Genomic tools were deployed successfully in groundnut improvement programme with the identification of QTL regions governing economically important traits that include leaf rust and LLS resistance (Khedikar *et al*., 2010 and Sujay *et al*., 2012). Markers that are closely linked or associated with genes/ QTLs for some important target traits have been identified in groundnut and are being utilized to transfer genes/QTLs to elite cultivars.

2.7 Marker-assisted selection (MAS)

MAS is a new paradigm in plant breeding. It involves the selection of genotypes carrying a desirable gene or gene combination, *via* linked marker(s). The introgression of disease resistance into elite popular cultivars is the most promising approach, provided resistant cultivars are available within the cultivated gene pool. Under conventional backcross breeding methods, recovery of all the recurrent parental genome would typically take 6-8 backcross generations (Allard, 1960 and Collard *et al*., 2005). Although significant strides have been made in crop improvement through phenotypic selections for agronomically important traits, considerable difficulties are often encountered during this process, primarily due to G × E interactions and resource intensive phenotypic selection.

Recent advances in crop genomics facilitate the identification of molecular markers associated with target trait(s) that can be deployed to select a superior line in a breeding programme.

The development of molecular markers and linkage maps has made it possible to identify the major genes/ QTLs responsible for foliar disease resistance in groundnut. Data from simulation studies suggested that at least two but possibly three or even four backcross generations can be saved by using markers (Mebrouk *et al*., 2005).

Molecular marker-aided selection involves screening segregating individuals for the presence or absence of the desired plant phenotype indirectly based on DNA banding pattern of linked markers on a gel or on autoradiogram depending on marker system. The rationale is that banding patterns reveal the parental origin of the bands in segregants at a given marker locus and hence indicate the presence or absence of specific chromosomal segments that carry the desired alleles. This increases the screening efficiency in breeding programmes. Concibido *et al*. (1996) defined MAS as an indirect selection method based on genotype rather than plant phenotype. They reported that MAS can be used to accelerate the rate at which new cultivars are developed. MAS may be especially useful in the early generations of pedigree selection or in the selection of characters that are difficult or expensive to measure such as drought. MAS could also be applied to individual plants in succeeding generations without the necessity of extensive progeny tests (Lande, 1992).

Molecular markers offer a suitable method for the introgression of resistance genes/QTLs from *Arachis* species to *A. hypogaea*, thereby facilitating selection of desirable progenies carrying the resistance genes in early and advanced generations of interspecific hybrids. Different DNA marker systems can be used to monitor chromosome segments in the breeding population. Microsatellite or SSR markers have become the assay of choice for genetic studies in *Arachis* because they are multiallelic, co-dominant, transferable among related species, polymerase chain reaction-based markers and usable in tetraploid genomes. Efforts by several research groups to develop microsatellite markers for groundnut have resulted in more than 15,000 SSRs (Pandey *et al.,* 2012). Breeding for foliar disease resistance in groundnut has now reached a stage where molecular markers can be successfully employed for genotypic selection, which could enhance the breeding efficiency.

The use of genomic resources coupled with marker-assisted selection provides a tool to develop improved genotypes for important traits. The advent and coupling of genomic tools like markers and marker-assisted selection (MAS) along with conventional breeding approaches enhance the precision and speed of developing groundnut cultivars with LLS and rust resistance.

'NemaTAM', the first root-knot nematode-resistant groundnut variety was bred using marker-assisted breeding (MAB), and it was released for cultivation in the USA (Simpson *et al*., 2003).

In the USA, MAS has been used for pyramiding nematode resistance and high oleic trait. 'Tifguard High O/L' was generated through three rounds of accelerated backcrossing using Tifguard as recurrent parent and progenies were selected with molecular markers for these two traits (Chu *et al*., 2011).

Wang *et al*. (2013) characterized four high oleic lines by full-length *ahFAD2* gene sequencing of PCR amplified products and used overlapped peaks for the selection of true hybrids.

The marker-assisted backcrossing (MABC) for high O/L ratio in peanut was demonstrated with a breeding line showing a high O/L ratio (YI-0311) and an elite line with medium O/L ratio ('Nakateyutaka') as a donor and backcross parents. Nine homozygous lines were identified from 205 BC_2F_2 plants for high O/L ratio (Koilkonda *et al*., 2013).

One QTL region on linkage group AhXV explaining up to 82.6% phenotypic variation for rust resistance was validated and introgressed from resistant cultivar 'GPBD 4' into three rust susceptible elite popular groundnut varieties (ICGV 91114, JL 24 and TAG 24) through MABC. The MABC approach employed a total of four markers including one dominant (IPAHM103) and three co-dominant (GM2079, GM1536, GM2301) markers present in the QTL region. Field evaluation identified 81 introgression lines (ILs) with improved rust resistance. Those ILs had significantly increased pod yields (56.0-96.0%) in infested environments compared to the susceptible parents (Varshney *et al*., 2014).

Sharanabasappa and Bhat (2015) initiated a program to improve JL 24, an elite variety for disease resistance by employing late leaf spot and rust resistance linked markers. They developed 4 backcross lines of JL 24 using the donor, GPBD 4 and identified four lines as superior for disease resistance and productivity.

Using allele-specific PCR and CAPS assays, Janila *et al*. (2016a) have introgressed the *ahFAD2A* and *ahFAD2B* mutant alleles in different high and low oil (%) containing groundnut genotypes. The high oleic lines (83) were developed in the genetic background of three cultivars (ICGVs 06110, 06142, and 06420).

Leal-Bertioli *et al*. (2015) identified the genomic genome regions associated with components traits for rust resistance. They developed RILs from a cross between two *Arachis* species, a susceptible *A. ipaënsis* (accession GKBSPSc 30076) and *A. magna* (accession GKSSc 30097), resistant to rust. QTLs for several components identified on linkage group B08. Twenty-two KASP markers were developed and validated for rust resistance in both diploid and tetraploids.

Sukruth *et al*. (2015) employed a set of four RILs, three backcross populations and synthetic tetraploids (developed from wild diploids) for validating LLS and rust resistance-linked markers. They identified the significant association of linked markers *viz*., GM2009, GM2301, GM2079, GM1536, GM1954 and IPAHM103 with rust resistance; GM1954, GM1009 and GM1573 markers with LLS resistance.

Seventy-eight RILs (F_9) from Gregory × Tifguard were used for genotyping with polymorphic SSR or SNP markers for root-knot nematode (RKN; *Meloidogyne arenaria*). SNP marker, AdSNP 584 detects two alleles at a single locus on linkage group A09 (Chu *et al*., 2016).

A total of one hundred thirty F_2 plants derived from cultivar Yueyou 92, the highly resistant to bacterial wilt (BW) disease, while cv. Xinhuixiaoli, highly susceptible to BW. Two QTLs (*qBW-1* and *qBW-2*) were detected for BW resistance located on linkage groups LG1 and LG10 and accounted for 21 and 12 % of the phenotypic variance. They developed 26 SNPs using the KASP genotyping method to screen for BW resistance (Zhao *et al*., 2017). In another study, Kolekar *et al*. (2017) conducted a marker-assisted backcrossing (MABC) programme to improve TMV 2 for LLS and rust resistance from the resistant parent GPBD 4.

They developed four lines and out of them two homozygous backcross lines TMG-29 and TMG-46 were found to be resistant to LLS and rust. These lines showed enhanced resistance to LLS and rust diseases (score of 3.0 for both) along with 71.0% and 62.7% increase in the pod yield per plot, respectively, over the check, TMV 2.

Marker-assisted selection was deployed in hybridization between the nematode-resistant cultivar Tifguard and high oleic cultivar Florida-07. The selected genotype TifNV-High O/L exhibited significantly higher yields in comparison to Georgia-06G, a widely grown susceptible cultivar tested in fields with nematode pressure. 'TifNV-High O/L' was developed resistance to the peanut root-knot nematode and tomato spotted wilt tospovirus with high oleic acid. This cultivar was released by the USDA-ARS and the Georgia Agricultural Experiment Station in 2014 (Holbrook *et al*., 2017).

Chopra *et al*. (2018) identified 15 definitive and 16 putative QTLs for petiole length, leaflet length and width, leaflet area, leaflet length/width ratio, main stem height, the presence of flowers on the main stem, and seed mass in 91 F_2 lines.

Whole-genome resequencing (WGRS) of RIL populations (Tifrunner × GT-C20) facilitated the fine mapping and candidate gene discovery for early leaf spot (ELS) and LLS, and Tomato spotted wilt virus (TSWV). Five SNPs were discovered associated with ELS and LLS QTLs using KASP assay (Agarwal *et al*., 2018).

2.7.1 Morphological evaluation of MAS and MABC derived material in groundnut

Morphological characterization continues to be the backbone of a varietal development program. Hence, it is indispensable in crop improvement program to evaluate the available variability of early and advance generations of breeding lines under field conditions to identify the superior and desirable traits of farmers and consumer preference. Field screening makes the information to percolate the crop improvement workers to exploit the genetic potential in the desired direction. A wide spectrum of variability is harboured over years for characterization and utilization in groundnut in cultivar improvement program.

Simpson *et al*. (2013) developed 'Webb' a high-yielding, high-oleic fatty acid, nematode-resistant groundnut cultivar that has a moderate level of resistance to *Sclerotinia blight*. The cultivar was developed in the Texas peanut breeding program and released by Texas AgriLife Research. Webb has performed well in 19 yield tests across five years, and it has good blanching and shelling characteristics.

Jakkeral *et al*. (2014) developed the recombinant inbred lines (F_6) generated by single seed descent (SSD) method from cross GPBD-5 × GPBD-4 and the rust-resistant plants were backcrossed to the recurrent parent (GPBD-5) to develop a backcross population (BC_1F_4). They observed a shift in correlation from negative (F_6) to significant positive direction (BC_1F_4) between pod yield per plant with plant height and between plant height with a number of primary branches. They concluded that the backcrossing of selected plants followed by advancing by SSD method is more rewarding than the single cross for improvement in the traits such as the number of primary branches, number of pods per plant, kernel yield per plant and shelling percent.

The MABC program was undertaken to improve an elite cultivar but foliar disease susceptible variety of groundnut JL 24, using the donor cultivar, GPDB 4. The backcrossed lines (BC_1F_4, BC_2F_3 and BC_3F_3) were evaluated for resistance to LLS and rust, and productivity traits. Four lines were identified resistant to LLS and rust and on par with JL 24 for the productivity traits (Sharanabasappa and Bhat, 2015).

Janila *et al.* (2016b) developed ILs in the genetic background of TAG 24, ICGV 91114 and JL 24 with major QTLs for rust (>80.0% PV) and LLS (67.9% PV) resistance. Six best ILs were selected with 39.0-79.0% higher mean pod yield and 25-8% higher mean haulm yield over their respective recurrent parents through field evaluation for two seasons.

Kolekar *et al*. (2017) developed TMV 2 derived backcross lines and evaluated segregating population (F_3, F_4, BC_1F_3 and BC_1F_4) for LLS, rust resistance, morphological features, and yield performance. Two homozygous backcross lines TMG-29 and TMG-46 showed enhanced resistance to LLS and rust diseases (score of 3.00 for both) along with 71.0% and 62.7% increase in the pod yield per plot, respectively, over the check, TMV 2.

2.8 Oil quality parameters in groundnut

Fatty acids are major components of triacylglycerols, phospholipids, and other complex lipids. Oilseeds like groundnut are rich in these lipids and their fatty acid components. The groundnut oil is one of the healthy cooking oil as the ratio of unsaturated fatty acids (UFA) to saturated fatty acids (SFA) is very high as compared to coconut and palm oil (Janila *et al*., 2016a).

The groundnut seed comprises around 50.0% oil, of which approximately 80.0% consists of oleic acid (36.0–67.0%) and linoleic acid (15.0–43.0%), a monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) respectively (Moore and Knauft, 1989).

Normal groundnut oil with high linoleic acid is vulnerable to oxidative rancidity, making it undesirable for human intake as it becomes thermodynamically unstable when heated at high temperature (Kratz *et al*., 2002) and reduces the shelf life of the oil and other groundnut products. Palmitic acid is known to cause an adverse effect on human health as it has been found to increase the risk of developing cardiovascular diseases (CVD). An omega-9 fatty acid, oleic acid, cuts down the risk of CVD by reducing low-density lipoprotein (LDL) levels in the blood (Calder, 2015), suppress tumorigenesis, and ameliorate inflammatory diseases (Yamaki *et al*., 2005 and Garcia *et al*., 2006).

A high oleic to the linoleic acid ratio (O/L ratio) is the most desired quality trait in groundnut as it increases shelf life to manufacturers and the health benefits to consumers. It is currently a high priority for groundnut breeders to introduce the high O/L trait into all groundnut cultivars and select the genotypes with a lesser amount of unfavorable fatty acids *i.e*. linoleic and palmitic acid. Despite the availability of high oleic acid sources in available germplasm, the exploitation of this trait was limited due to non-availability of suitable equipment for estimating fatty acid profiles and also could be due to lack of awareness about the importance of this trait. The identification of natural mutant, F 435 (~80% oleic acid) (Norden *et al*., 1987), which lacks functional fatty acid desaturase (FAD) enzyme was breakthrough in groundnut breeding to breed high oleic acid and reduced linoleic acid content in kernels. Later on, F 435 derived line SunOleic 95R was used as a donor line for improvement of groundnut for high oleic acid content (Gorbet and Knauft, 1997). With that intent, groundnut breeding program at ICRISAT in collaboration with NARS partners has taken initiative to introduce high O/L trait into elite cultivars of semi-arid tropics (Janila *et al*., 2016a).

The highlights on the importance of groundnut with high oleic content are as follows.

2.8.1 Importance of groundnut featuring high oleic acid

1. Painty and cardboard flavors were more stable for HOA groundnuts than normal after 6 weeks of storage. Shelf life estimated from sensory data was twice as long in HOA groundnuts (Braddock *et al.,* 1995).
2. Low fat-monounsaturated rich diets containing high-oleic groundnuts improve serum lipoprotein profiles (O'Byrne *et al.,* 1997).
3. High oleate groundnut lines were rich in α-tocopherol, phytosterols, and phosphatidylcholine (Jonnala *et al*., 2006).
4. Oleic acid and groundnut oil high in oleic acid reverse the inhibitory effect of insulin production of the inflammatory cytokine TNF-α both in *in vitro* and *in vivo* systems (Vassiliou *et al.,* 2009).
5. Craft *et al*. (2010) reported that high oleic groundnuts had the greatest (78.5%) in free ρ-coumaric acid levels after oil roasting.
6. The consumption of high-oleic groundnuts may help to reduce the risk of endotoxemia and metabolic disorders (Moreira *et al.,* 2014).
7. Regular intake of high-oleic groundnuts improves fat oxidation and body composition in overweight men pursuing an energy-restricted diet (Alves *et al.,* 2014).
8. Barbour *et al.* (2017) recommended high oleic groundnuts to overweight middle-aged adults for cerebrovascular and cognitive benefits.

2.8.2 Molecular studies on high oleic acid content in groundnut

An understanding of the molecular mechanisms that are responsible for increased oleic acid accumulation would open avenues to alter groundnut fatty acid composition and allow detection of polymorphic regions which can be used for MAS. In this view, many studies have reported the improvement of O/L ratio in groundnut. Inheritance of high oleic acid is under simple genetic control (Moore and Knauft., 1989). The inheritance of oleic acid in Virginia/Spanish type groundnut type was reported to be controlled by two loci but modifiers and additional epistatic interactions may be occurring (Isleib *et al.,* 1996).

Generation means analysis for oil quality traits indicated the predominant role of additive and additive × additive gene action in the genetic control of the fatty acid content and quality parameters (Aruna and Nigam, 2009 and Singkham *et al.,* 2012).

The wild-type homoeologous genes (*FAD2A* and *FAD2B*) encode microsomal oleoyl-PC desaturase also known as Δ12 fatty acid desaturase located on linkage group A09 in A-genome and linkage group B09 in B-genome, respectively (Moore and Knauft, 1989). This enzyme is responsible for catalyzing the conversion of oleate to linoleate (Ray *et al*., 1993) by the addition of a second double bond which generates a polyunsaturated fatty acid from a mono-unsaturated fatty acid (Schwartzbeck *et al.,* 2001).

The open reading frames (ORF) for *FAD2A* and *FAD2B* are 99 percent identical and consist of 1,140 bp, encoding 379 amino acids with no introns in the coding sequence (Jung *et al.,* 2000 and Lopez *et al.,* 2000). Comparisons of the coding sequences from the high and low oleic acid genotypes revealed several SNPs. Sequence information was used to identify SNPs from genomic DNA for the Δ12 FAD of Spanish-type groundnut cultivars (Lopez *et al.,* 2000). Two of the SNPs apparently was associated with the high oleate trait. F435-derived high oleic groundnut cultivars contain two key mutations within the Δ12 fatty acid desaturase gene which include a 1 bp substitution of G:C→A:T at 448 bp of ORF in the A genome, resulting in the replacement of aspartic acid with asparagine and a 1 bp insertion of A →T at 442 bp of ORF in the B genome. The "A" insertion shifts the amino acid reading frame, probably resulting in a truncated, inactive protein (Ohlrogge and Browse, 1995., Lopez *et al.,* 2000 and Yu *et al.,* 2008) leading to reduced activity of desaturase.

However, selection for seed quality is practised only in advanced breeding lines, as the biochemical estimation for these traits in segregating populations is high resource demanding, cumbersome and time-consuming. Two homeologous genes, *ahFAD2A* and *ahFAD2B* have been found to control the oleoyl-PC desaturase activity and cleaved amplified polymorphic sequences (CAPS) markers were developed to differentiate mutant and wild-type *ahFAD2A* alleles (Chu *et al.,* 2009). Further, based on the previous study a simple PCR assay for detection of *FAD2* alleles on both genomes was developed by designing allele-specific primers and altering PCR annealing temperatures.

This assay was successfully used for detecting *FAD2* alleles in groundnut. For oil and fatty acid composition, near infrared reflectance spectroscopy (NIRS) was used to determine the fatty acid composition of PCR-assayed genotypes (Janila *et al.,* 2016a).

These mutations lead to the accumulation of more oleic acid and decreasing linoleic and palmitic acid ensuring oil quality improvement by increasing shelf life. Keeping in mind the preferences of consumers and profitability to growers, groundnut breeding mainly focuses on developing improved varieties with desirable levels of oil content and other fatty acids, especially oleic, linoleic, and palmitic acids. Successful introgression of the FAD mutant alleles from SunOleic 95R with increased oleic acid has already been demonstrated using genomics-assisted breeding (GAB) approaches such as MABC and MAS in both high and low oil containing genotypes (Chu *et al*., 2011 and Janila *et al.*, 2016a).

2.8.3 Estimation of oil quality parameters in groundnuts

The modification of the fatty acid composition of seed oil has been an important objective in groundnut breeding. In the process of cultivar improvement for quality traits, a large number of breeding lines and individuals of segregating population need to be screened for quality parameters like oil content, fatty acids, protein etc. The fatty acid composition is usually determined by gas-liquid chromatography (GLC) equipped with a flame ionization detector (GLC-FID) and a capillary column. Fatty acids must be converted to fatty acid methyl esters (FAMEs) for GLC analysis. However, these biochemical methods require multi-step sample preparation, they are time-consuming, expensive, labor-intensive, and also destructive; therefore, they are not feasible for selecting superior lines from a number of groundnut lines in the breeding pipeline. Storing the groundnuts with excessive moisture content increases the risk of microbial growth and aflatoxin contamination during storage. Therefore, estimation of moisture content in groundnut is important prior to storage and processing. Hence, for plant breeding programs that are focused on the modification of fatty acids, oil and protein content of groundnut kernels, there is a need for simple, non-destructive, economical and fast screening methods. The deployment of the rapid and non-destructive method enables the groundnut breeders to make a decision on hybridization and selections based on kernel quality parameters.

Near-infrared reflectance spectroscopy (NIRS) offers a simple, non-destructive, economic and fast screening tool for estimation of quality parameters by utilizing the near-infrared rays (750–3000 nm) of the electromagnetic spectrum (Sundaram *et al*., 2009). NIRS tool has proven its applicability in quantitative measurements of protein, carbohydrates, moisture, oil, fatty acids, acidity, total soluble solids, seed weigh (Vollmann *et al*., 2017). Due to its quick assay (about 80 seconds per sample), favorable economics, absence of sample preparation and chemicals, the NIRS has been successfully used to estimate bio-chemical compounds in several oilseeds crops such as sunflower, sesame, and soybean (Fassio and Cozzolino, 2004., Kim *et al*., 2006 and Ferreira *et al*., 2014). NIRS tool has proven its applicability in quantitative measurements of moisture content, oil content, fatty acids, protein (Sundaram *et al*., 2009).

MATERIAL AND METHODS

Chapter III

MATERIAL AND METHODS

The materials used and methods adopted in the present study for hybridization; biochemical, molecular, pathological experiments; statistical methods followed for data analysis are described in this chapter.

3.1 Experimental site

In the present study, hybridization and generation advancement was carried out in the greenhouse and experimental fields at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Telangana, India. The field evaluation of the backcrossed lines and parents was carried out during the summer season 2018 at red campus West 18 field-block (17.51º North latitude and 78.27º East longitude). The soil type of the experimental block was Alfisols (Patancheru Soil Series; UdicRhodustolf) with a pH ranging from 7.0 to 7.5. The air temperature ranged between 22° C to 36° C and relative humidity varied between 34 to 87% during summer 2018.

3.2 Plant material

A popular groundnut cultivar, Kadiri 6 (K 6) developed at Agricultural Research Station, Kadiri of Acharya N. G. Ranga Agricultural University, Andhra Pradesh in 2005 was used as recipient parent. For foliar disease resistance, breeding line ICGV 13193 derived from the cross (ICGV 91114 × GPBD 4) with a pedigree of {ICGV 91114-P1 × [ICGV 91114-P1 × (ICGV 91114-P1 × GPBD 4-P1_13-1)]} was utilized as a donor parent. ICGV 13193 is late leaf spot (LLS) and rust resistant line with a score of 2.0 and 1.0 respectively at 75 days after sowing (DAS). For high oleic trait, ICGV 15033 with a pedigree of [(ICGV 06420 × Sun Oleic 95R) F_2P402-P1-P1-B1-B1] with an oleic acid content of ca.= 82% was used as a donor line. The features of parental lines used in the crossing program are presented in Table 3.1.

Table 3.1. Details of parental lines used in the marker-assisted breeding program

S. No.	Parental line	Cross details	Features	Reference
1.	Kadiri 6	(JL 24 × Ah-316/S)	Short duration and popular cultivar among the farmers	Reddy *et al*. (2015)
2.	ICGV 15033	(ICGV 06420 × SunOleic 95R)	High oleic acid content (ca. 82.00%)	Janila *et al*. (2016a)
3	ICGV 13193	(ICGV 91114 × GPBD 4)	Resistant to LLS and rust with disease score of 2 and 1 respectively at 75 DAS	Janila *et al*. (2016b)

3.3 Raising the crop and hybridization scheme

The parental lines K6, ICGV 15033 and ICGV 13193 were sown on ridges with the row length of 4 m for each genotype. Row-to-row and plant-to-plant spacing was 60 cm and 10 cm respectively. The groundnut hybridization technique described by Nigam *et al*. (1990) was used in the current study. The breeding scheme used to improve K 6 is described in Figure 3.1.

Two independent crosses were made with K 6 serving as a pistillate parent and ICGV 15033, and ICGV 13193 as donor parents to generate F_1s during rainy 2016. These F_1s were grown in the greenhouse during post-rainy 2016-17 and were genotyped with allele-specific markers for high oleic acid and linked SSR markers for rust and LLS to identify true F_1 plants. The true F_1 plants carrying mutant *ahFAD2* alleles, and QTLs for LLS and rust were intercrossed to pyramid LLS, rust and high oleic traits in a single line. The obtained F_1s were genotyped with diagnostic SNP markers to identify true intercrossed F_1s. The pyramided F_1 plants positive for six SNPs were used as a pollen parent and recurrent parent K 6 as a pistillate parent to make backcross during summer 2017 to obtain BC_1F_1 seeds. The BC_1F_1 seeds were harvested and planted in post-rainy 2017-18. The BC_1F_1 plants were genotyped with SNP markers and positive plants were allowed to self. The harvested BC_1F_2 seeds were sown in the field during Summer 2018 and evaluated for morpho-agronomic traits and oil quality parameters. The qudrafoliate leaf collected from BC_1F_2s were used to screen for LLS and rust resistance using a detached leaf assay.

3.4 Marker-assisted selection for late leaf spot, rust, and high oleic traits

3.4.1 Genotyping by gel-based molecular markers

To track QTL region, earlier reported SSR markers IPAHM103, GM2301, GMRQ786, and GMRQ843 were used for rust, SEQ8D09 and GMLQ975 were used for LLS (Khedikar *et al*., 2010., Sujay *et al*., 2012 and Pandey *et al*., 2017). The SSRs markers (Table 3.2) are associated with rust-resistant QTLs with 42.7-83.6% phenotypic variation explained (PVE) and LLS resistant QTLs with 9- 63.1% PVE.

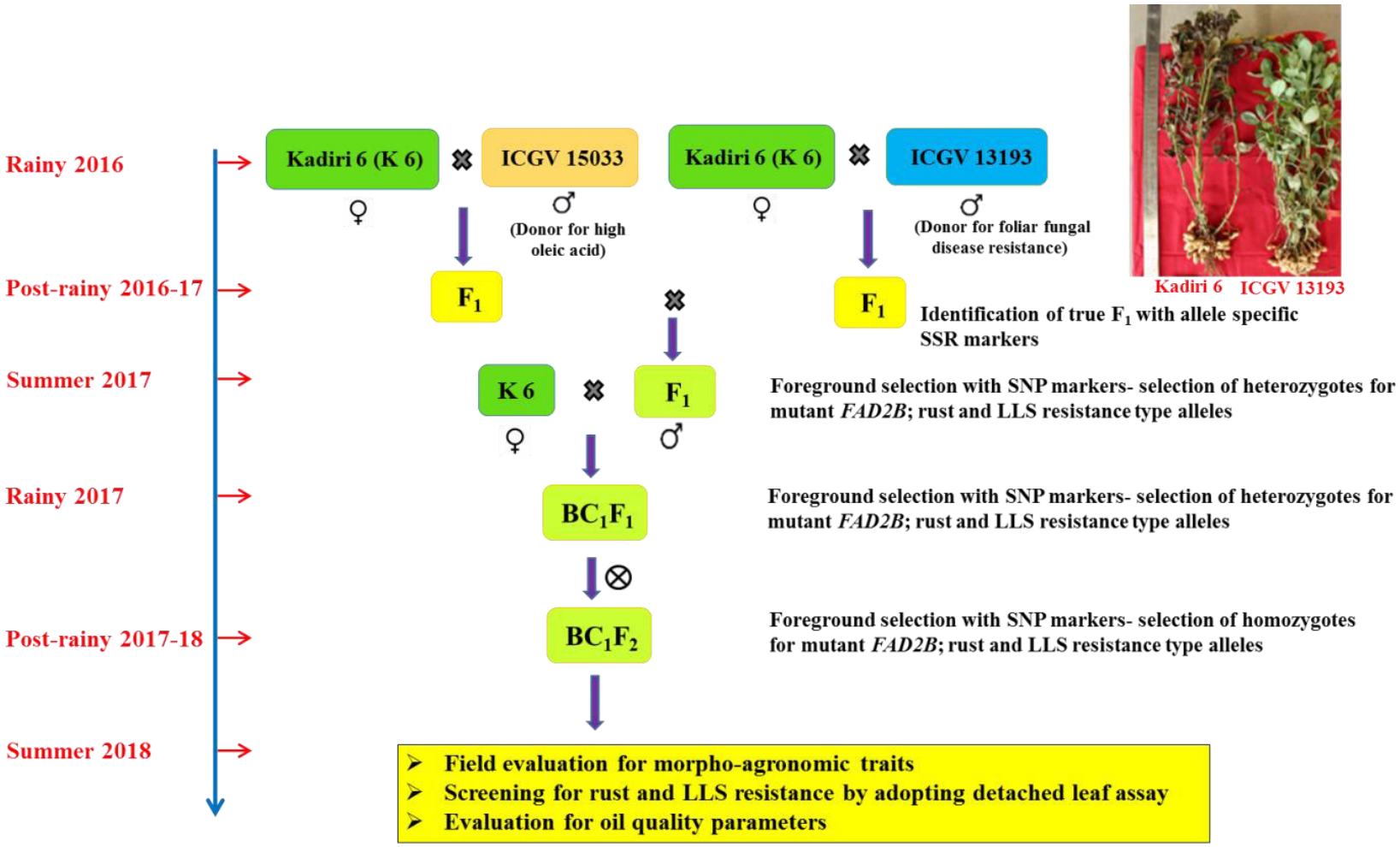

Figure 3.1 Marker-assisted backcross breeding scheme for improvement of foliar fungal disease resistance and oil quality in groundnut *cv*. Kadiri 6.

Table 3.2. List of SSR markers used for genotyping of late leaf spot and rust resistant alleles

Trait	Linked markers	Linkage group	Sequence (5′-3′)	Annealing temperature (°C)	References
Rust	IPAHM103	A03	FP: GCATTCACCACCATAGTCCA RP: TCCTCTGACTTTCCTCCATCA	60	Khedikar *et al*. (2010)
Rust	GM2301	A03	FP: GTAACCACAGCTGGCATGAAC RP: TCTTCAAGAACCCACCAACAC	60	Sujay *et al*. (2012)
Rust	GMRQ786	A03	FP: AACATTGTAACACTCACCTGGCTA RP: TCATGCTTGAACTGTGCCTC	59	Pandey *et al*. (2017)
LLS	GMLQ975	A03	FP: GGTATCATGATGAATTTTTAGAAGACTAGG RP: GAAATTTGGCTTTGGGTTCA	59	Sujay *et al*. (2012)

FP: Forward primer and RP: Reverse primer

The F435, first high oleic natural groundnut identified has substitution (G:C to A:T) and insertion (A:T) of one base pair in *ahFAD2A* and *ahFAD2B* genes respectively (Chen *et al*., 2010). The allele-specific markers were used to identify *ahFAD2A* and *ahFAD2B* alleles in F_1 generation (Table 3.3).

3.4.2 Genotyping by high throughput SNP markers

Recently, groundnut breeding at ICRISAT is taking the assistance of Intertek Private LTD, Hyderabad for high throughput plant genotyping (HTPG). The target traits *viz*., oleic acid, rust, and LLS resistance was selected for Kompetitive allele-specific PCR (KASP) assay. The SNP markers developed through QTL-seq (Pandey *et al*., 2017) was shared with Intertek for this assay. The leaf discs were collected and sent to Intertek for SNP genotyping which involved a set of six diagnostic SNP's in a panel for the three targeted traits.

KASP^{TM} genotyping are based on competitive allele-specific PCR and enable biallelic scoring of single nucleotide polymorphisms (SNPs) and insertions and deletions (Indels) at a specific locus. The SNP-specific KASP Assay mix and the universal KASP Master mix are added to DNA samples, a thermal cycling reaction is then performed, followed by an end-point fluorescent read. Bi-allelic discrimination is achieved through the competitive binding of two allele-specific forward primers, each with a unique tail sequence that corresponds with two universal FRET (fluorescence resonant energy transfer) cassettes; one labelled with FAM™ dye and the other with HEX™ dye. A set of six validated diagnostic SNP markers (Table 3.4); one SNP for *ahFAD2B*, and five SNPs for LLS and rust was used for genotyping backcrossed population and identifying pyramided lines. From each backcrossed plant two small leaf discs were excised, collected in 96 well plates and sent to Intertek Pvt. Ltd., Hyderabad for genotyping.

3.5 DNA extraction, polymerase chain reaction and electrophoresis

3.5.1 DNA extraction

DNA was isolated from the young leaves of F_1s, backcross progenies and parents by following the modified cetyl trimethyl ammonium bromide (CTAB) method. Young leaf tissues (50-100 mg) of 10-15 days old seedlings were placed in 12 × 8-well strip tube with strip cap (Marsh Biomarket, USA) in a 96 deep-well plate together with three 4 mm stainless steel grinding balls.

Table 3.3. Details of allele-specific markers used in screening for fatty acid desaturase 2 (*ahFAD2*) alleles

Target allele	Marker name	Sequence	Annealing temperature (°C)	Mutant allele (bp)	Wild-type allele (bp)	References
FAD2A	F435-F	Forward: ATCCAAGGCTGCATTCTCAC Reverse (SUB): TGGGACAAACACTTCGTT	59	203	290	Chen *et al*. (2010)
FAD2B	F435	Forward: ATCCAAGGCTGCATTCTCAC Reverse (INS): AACACTTCGTCGCGGTCT	59	195	290	Chen *et al.* (2010)

Table 3.4. Selected SNPs associated with late leaf spot, rust resistance and high oleic acid traits used for high throughput genotyping

SNP ID Intertek	SNP code	Trait Category	Selected SNP code	Genomic position (bp)	Target allele (donor type)	Alternate allele
snpAH0002	GKAMFAD2B	Oil quality	1	B sub-genome	A	-:-*
snpAH0015	GKAMA03QR786	LLS and rust resistance	6	133497786	T	A
snpAH0017	GKAMA03QR517	LLS and rust resistance	7	131739517	A	C
snpAH0018	GKAMA03QR796	LLS and rust resistance	8	131937796	G	A
snpAH0021	GKAMA03QR661	LLS and rust resistance	9	133527661	C	G
snpAH0026	GKAMA03GR173	LLS and rust resistance	10	134613173	G	C

* -:- Null allele

For each sample, 450 µl of preheated (at 65º C for half an hour) extraction buffer (100 mM Tris-HCl (pH-8), 1.4 M NaCl, 20 mM EDTA, CTAB (2-3% w/v), mercaptoethanol) was added and sealed with caps. Samples were homogenized in a GenoGrinder 2000 (Spex CertiPrep, USA), at 500 strokes/min for 3 times at 1.5 min interval. The plates were incubated at 65º C in a water bath for 20-30 min with proper mixing at periodical intervals. For each sample, 450 µl of chloroform-isoamyl alcohol (24:1) was added and mixed thoroughly by gentle inverting. The plates were centrifuged at 5,500 rpm for 10 min and the aqueous layer (300 µl) was collected in fresh tubes. The chilled isopropanol (stored at –20º C) (~250 µl) of was added to the collected aqueous layer and mixed thoroughly by inverting. The plates were centrifuged at 5,000 rpm for 15 min and the supernatant was discarded from each sample. The pellet was suspended in ethanol (70%) centrifuged at 6,000 rpm for 10 min and the supernatant was discarded. The pellet was allowed to dry for 20 min. For RNase treatment, 200 µl low salt TE (10 mM Tris EDTA (pH-8)) and 3 µl RNase (10 mg/ml) was added to each sample and incubated at 37 ºC for 30 min.

DNA was quantified by loading 1 µl on the 0.8% agarose gel (120 ml) containing 10 µl ethidium bromide (10 mg/ml) run at 80V for 30-45 minutes. DNA was normalized to 5 ng/µl concentration by dilution. The agarose gel was documented under UV transilluminator. DNA quality and quantity were also measured using Nanodrop (Shimadzu UV160A, Japan).

3.5.2 Genotyping for FDR

Touchdown PCR programme was used for screening SSR (Table 3.2) of FDR QTLs. The PCR (Polymerase Chain Reaction) mix consisted of 2-5 ng of DNA, 2 pmol of M13 labelled forward (F), 5 pmol of reverse (R), 2 mM MgCl2, 2 mM dNTPs, 0.1 U of Taq DNA polymerase (Kappa Taq) and 1X PCR buffer. A standardized touch down PCR program with 5 minutes initial denaturation, followed by 5 cycles of 94º C for 20 secs, 65º C for 20 secs and 72º C for 30 secs with 1º C decrement for every cycle, followed by 40 cycles of 94º C for 20 secs, constant annealing temperature of 59º C and 72º C for 30 sec ending with extension for 20 minutes at 72º C. PCR products were resolved on 2% agarose gel for confirming the amplification. In SSRs, forward primers were dye-labelled with FAM, VIC and NED which are detected as blue, green and black colour peaks respectively (Applied Biosystems) upon capillary electrophoresis. The PCR products were denatured and capillary separated with ABI 3700 automatic DNA

sequencer (Applied Biosystems, USA) and GeneMapper Software v (Applied Biosystems) was used to analyse the peak patterns.

3.5.3 Genotyping of high oleic acid

Genotyping by allele-specific markers: Allele-specific markers are developed by Chen *et al*. (2010), were used in the identification of mutant *ahFAD2A* and *ahFAD2B* alleles (Table 3.3). The touchdown PCR program was used for allele-specific markers as detailed in chapter 3.5.2. The PCR products were resolved on 2% agarose gel for confirming the amplification The 203 bp fragment with substitution (G:C to A:T) in *ahFAD2A* was amplified by F435-F and F435SUB-R while the 195 bp fragment with insertion mutation (A:T insertion) of *ahFAD2B* was amplified by F435-F and F435INS-R. In order to avoid false positives, primer combination of F435-F and F435-IC-R was used to amplify the 250 bp of both *ahFAD2A* and *ahFAD2B* capturing the whole fragment. The sample under study was amplified by all these three primer combinations. During the agarose gel electrophoresis, post PCR mixture is made *i.e*., PCR product of F435-SUB is mixed with F435-IC and loaded to identify the mutant in A sub genome, while in case of B subgenome post PCR mixture of F- 435-F and F435-INS were mixed and loaded.

In both the cases, the two bands were observed i.e., one internal control and another mutated band of either *ahFAD2A* or *ahFAD2B.*

3.6 Phenotypic evaluation of backcross progenies under field condition

The progenies BC_1F_2 were raised in experimental plots of during summer 2018 (April-July). The progenies were raised in 2 m rows with a spacing of 30 cm between rows, and 10 cm from plant to plant in a row. Recommended agronomic management practices were adopted to raise a healthy crop. Fertilizers included the basal application of 60 kg phosphorus pent-oxide (P_2O_5), and gypsum @ 400 kg/ha at peak flowering stage. Seed treatment was done with mancozeb @ 2 g/kg seed, and imidachloprid @ 2 ml/kg seed. To manage weeds, pendimethalin @ 1 kg active ingredient/ha was used as a pre-emergence application to the soil. All the plants in each progeny row were harvested at maturity and dried in the field for two days before stripping the pods from plants. The stripped pods were cleaned for soil particles and dried further under shade to bring down the moisture content. Fully matured pods from each sample were shelled to obtain kernels for biochemical analysis.

3.6.1. Morpho-agronomic characters

The morpho-agronomic traits were recorded to check the genetic potential of the BC_1F_2s for various yield-related traits which may be of use for further utilization through hybridization and selection. The following traits were recorded by following the groundnut descriptor (ICRISAT and IBPGR, 1992).

1. Days to 50 flowering: Number of days taken from the date of sowing to day on which each progenies flowered 50 percent was recorded.
2. Plant height: The height of the plant was measured in centimeters (cm) from the ground level to the tip of the main stem at the time of harvest.
3. Pods per plant: Number of pods per plant was counted at the time of harvest.
4. Pod yield per plant: Total weight of dried and cleaned pods obtained from each plant was recorded and expressed as g/plant.
5. Seed yield per plant: The mass of total kernels obtained from the single plant was recorded and expressed as g/plant.
6. Shelling outturn: Shelling outturn was calculated by weighing the kernels from a unit weight of pods obtained from single plant and expressed in percentage.
7. Pod length (mm): The length of five dried and cleaned pods were measured using Vernier calipers and recorded in mm.
8. Pod width (mm): The width of five dried and cleaned pods were measured using Vernier calipers and recorded in mm.
9. Pod constriction: Randomly selected five pods were observed for the presence of constriction. It was categorized as none (0), slight (S), moderate (M), deep (D) and very deep (D).
10. Pod reticulation: Randomly selected five pods were observed for the presence of reticulation. It was categorized as none (0), slight (S), moderate (M) and deep (D).
11. Pod beak: Pod beak was recorded in five randomly selected pods. The data was recorded as absent (0), slight (S), moderate (M), prominent (P) and very prominent (VP).

12. Pod ridges: Pod beak was recorded in five randomly selected pods. The data was recorded as absent (0), slight (S), moderate (M) and prominent (P).

3.7 Screening for late leaf spot and rust resistance using detached leaf assay

A set of 348 BC_1F_2 progenies along with resistant checks *viz*., GPBD 4, ICGV 86855 (CS 16) and ICGV 13193 and susceptible controls Kadiri 6, TAG 24, JL 24 and TMV 2 were selected for the foliar fungal disease screening study using the detached leaf method (Foster *et al*., 1980). The fully expanded quadrifoliate leaves (third or fourth from top) from 45 days old plants were excised through pulvinus. The excised leaves were washed with deionized water and used as an explant.

The mixture of sand: vermiculite mixture (1:1 v/v) was prepared and steam sterilized at 15 lbs pressure and 121° C for 1 h in two cycles of the autoclave. The experiment was conducted in two replications by following a completely randomized design (CRD). The excised explants were planted in sterile culture (4 cm thick sand) in plastic trays (40 cm × 30 cm) and sprayed with LLS and rust inoculum (30,000 spores ml^{-1}). The trays were covered with plastic bags and incubated in the growth chamber at a temperature of 24º C and 85% relative humidity. Water was sprayed on to the leaves once daily up to 8 days after inoculation.

Disease development was determined every alternate day from 5 to 40 days after inoculation (DAI). Data on the following parameters were recorded.

3.7.1 Components of resistance for late leaf spot

(a) The incubation period (IP): IP is defined as days from inoculation to the appearance of the first lesion. It is recorded on each leaf every alternate day from 5 days to DAI.

(b) Latent period (LP): LP is defined as days from inoculation to the appearance of the first sporulating lesion. It is recorded on each leaf every alternate day from 5 to 37 DAI.

(c) Lesion number (LN): LN was recorded on each leaflet every alternate day from 5 to 21 DAI and on 30 DAI.

(d) Leaf area damage (LAD): The percent LAD was assessed by comparing the leaves with diagrams depicting leaves with a known percentage of their areas affected (Hassan and Beute, 1977). It was measured every alternate day from 5 to 21 DAI and on 30 DAI.

(e) Lesion diameter (LD): LD is the average diameter of five randomly selected lesions on each leaflet. It was measured at 25 DAI using Vernier caliper under 20 × magnifying lens and the data was recorded in mm.

3.7.2 Components of resistance to rust

(a) The incubation period (IP): IP is defined as days from inoculation to the appearance of a first pustule. It is recorded on each leaf every alternate day from 5 days to DAI.

(b) Latent period (LP): LP is defined as days from inoculation to the appearance of the first sporulation of pustule. It is recorded on each leaf every alternate day from 5 to 37 DAI.

(c) Leaf area damage (LAD): The percent LAD was assessed by comparing the leaves with depicting leaves with a known percentage of their areas affected. It was measured every alternate day from 5 to 21 DAI and on 30 DAI.

(d) Sporulation index: Sporulation index was recorded on a 1 to 9 scale where 1 = no sporulation and 9 = dense sporulation (91–100% of the uredium full with urediniospores and ruptured).

(e) Disease scores (on 45 DAI) due to rust were recorded as described for LLS. The experiments were terminated at 44 DAI as the leaves of many genotypes were completely weathered due to rust.

Disease score for rust and LLS diseases were recorded at 45 and 70 DAS in each plot on a modified 1-9 scale (Table 3.5 and 3.6), as given by Subrahmanyam *et al*. (1995).

Table 3.5. A Modified 9-point scale for late leaf spot screening of groundnut

Disease score	Phenotype description	Disease severity*
1	No disease	0
2	Lesions present largely on lower leaves; no defoliation	1-5%
3	Lesions present largely on lower leaves, very few on middle leaves; defoliation of some	6-10%
4	Lesions present on lower and middle leaves but severe on lower leaves; defoliation of some leaflets evident on lower leaves	11-20%
5	Lesions present on lower and middle leaves, over 50% of defoliation of lower leaves	21-30%
6	Severe lesions on lower and middle leaves; lesions present but less severe on top leaves; extensive defoliation of lower leaves; some defoliation on middle leaves	31-40%
7	Lesions on all leaves but less severe on top leaves; defoliation of all lower and middle	41-60%
8	Defoliation of all lower and middle leaves; severe lesions on top leaves evident.	61-80%
9	Almost all leaves defoliated, leaving bare stem; some leaflets may remain but show severe leaf spot	81-100%

*Percentage leaf area damaged by LLS

Table 3.6. A Modified 9-point scale for rust used for screening of groundnut

Disease score	Phenotype description	Disease severity* (%)
1	No disease	0
2	Pustules sparsely distributed, largely on lower leaves	1-5%
3	Many pustules on lower leaves, necrosis evident, very few pustules on middle leaves	6-10%
4	Number of pustules on lower and middle leaves, severe necrosis on lower leaves	11-20%
5	Severe necrosis of lower and middle leaves, pustules may be present on top leaves but less severe	21-30%
6	Extensive damage to lower leaves, middle leaves, necrotic with a dense distribution of pustules, pustules on	31-40%
7	Severe damage to lower and middle leaves, pustules densely distributed on top leaves	41-60%
8	100 percent damage to lower and middle leaves, pustules on top leaves	61-80%
9	Almost all leaves withered, bare stems seen	81-100%

* Percentage leaf area damaged by rust

3.8 Biochemical analysis for oil content and quality traits in backcross lines

The oil, protein content and fatty acid composition were estimated using NIRS (model XDS RCA, FOSS Analytical AB, Sweden, Denmark). Earlier the calibrations for estimation of fatty acids were done using gas chromatography (GC). Similarly, calibration for oil content was done using Soxhlet estimates on 142 genotypes with oil content ranging from 40% to 57%. The regression coefficient (R^2) value for predicting oleic and linoleic acid using the calibration equation was 0.96, while 0.80 for palmitic acid and 0.89 for oil content. Seeds obtained from the single plant were scanned in a rectangular cup.

3.9 Statistical analysis

Statistical analysis was done using the software package Windostat Version 8.1 and Microsoft Excel, 2016.

3.9.1 Mean and range: Mean was calculated for a set of the BC_1F_2 population by adding the observed value from the progenies and dividing the sum by a total number of observations. Difference between the minimum and maximum values observed for each trait was calculated as a range.

3.9.2 Variance: It measures the variability and defined as the average of the square of deviations from the mean.

3.9.3 Standard Deviation (SD): It is a dispersion of individual values (X) around the population mean.

3.9.4 Standard Error of Mean (SEm ±): It is a dispersion of population mean around the estimated population mean.

3.9.5 Coefficient of variation (CV): The coefficient of variation is the best statistical method to compare the amount of variation present in the population for different traits. Different traits are represented by different units so that by converting units of all characters on the same scale, we can compare the amount of variation in terms of CV which is expressed as percentage ratio.

RESULTS AND DISCUSSION

Chapter IV

RESULTS AND DISCUSSION

Groundnut is one of the most important oilseeds and food crops in the arid and semi-arid regions of the world including many Asian and African countries. The late leaf spot (LLS) and rust are the major foliar diseases and they often occur together leading to 50-70% losses in the pod and haulm yield. Hence, the development of groundnut lines resistant to LLS and rust is an economically and environmentally sustainable way. Many wild *Arachis* species exhibit resistant to LLS and rust and are used to develop interspecific derivatives (Gorbet *et al*., 1987., Singh *et al*., 2003., Gowda *et al*., 2002 and Nigam, 2015). Even though, wild *Arachis* species harbour a high level of resistance to these diseases, the introgression from wild species is challenging due to cross-compatibility barrier and linkage drags like undesirable pod and kernel features and delayed maturity. The present study attempted the marker-assisted breeding to combine LLS and rust resistance, and high oleic traits in Kadiri 6 (K 6), a popular cultivar in India. The marker-assisted backcross breeding approach was adopted to derive BC_1F_2 progenies and these progenies were phenotyped for morpho-agronomic and high oleic acid traits, and LLS and rust resistance under artificial disease epiphytotic condition using detached leaf assay.

The results and discussion of the present study are presented under the following headings:

4.1 Introgression of late leaf spot and rust resistance QTLs into Kadiri 6 cultivar

4.2 Introgression of alleles for high oleic acid trait into Kadiri 6 cultivar

4.3 Combining QTLs for late leaf spot, rust resistance and mutant *ahFAD2* alleles in Kadiri 6 cultivar

4.3.1 Kompetitive Allele Specific PCR (KASP) based SNP assay for zygosity test

4.4 Phenotypic evaluation of backcrossed lines for late leaf spot and rust resistance

4.4.1 Late leaf spot

4.4.2 Rust

4.5 Evaluation of backcrossed lines for morpho-agronomic and high oleic acid traits

4.5.1 Morpho-agronomic traits

4.5.2 Kernel quality traits

4.5.3 Superior segregants for late leaf spot and rust resistance, agronomic, and kernel quality traits

4.1 Introgression of late leaf spot and rust resistance QTLs into Kadiri 6 cultivar

DNA markers offer significant advantages through genotypic based selection for the desired trait early in the crop stage enabling early generation selection to speed up the breeding cycle. This helps in resource optimization and increases the efficiency of selection leading to an increase in the genetic gain. Hence, several efforts have been made to identify genes and to map the QTLs for LLS and rust resistance in groundnut. The QTL regions governing LLS and rust resistance were mapped on A03 chromosome of resistance line GPBD 4 derived population. The QTL regions on A03 was further resolved to 3.06 Mb (131.60- 134.66 Mb) genomic region on the A03 pseudomolecule of A genome (Pandey *et al*., 2017), which consisted ~25 putative candidate genes associated with LLS and rust resistance. Some of these markers were validated (Yeri et al., 2014 and Sukruth et al., 2015) and also used for developing LLS and rust resistant introgression lines (ILs) using marker-assisted backcrossing (MABC) approach (Varshney *et al*., 2014., Sharanabasappa and Bhat, 2015 and Janila *et al*., 2016b).

To combine LLS and rust resistance and early maturity in groundnut, Janila *et al*. (2016b) developed ILs in the genetic background of three popular cultivars, TAG 24, ICGV 91114 and JL 24. These ILs possess major QTLs for rust with >80.0% phenotypic variance (PV) and LLS (67.9% PV) resistance sourced from GPBD 4. Six best ILs were selected with 39-79% higher mean pod yield and 25-89% higher mean haulm yield over their respective recurrent parents through field evaluation for two seasons. Among these lines, ICGV 13193 derived from the cross (ICGV 91114 × GPBD 4) was utilized as a donor source for LLS and rust resistance in the present study.

The donor parent ICGV 13193 was crossed with K 6 during rainy season 2016 to produce 210 F_1 seeds. In the post-rainy, 2016-17, the seeds were sown in the greenhouse to raise 174 F_1 plants and tested for hybridity with SSR markers linked to rust (IPAHM103, GM2301, and GMRQ786) and LLS (GMLQ975) resistance (Table 4.1). A total of 128 plants (74%) were true hybrids carrying the target alleles for rust and LLS in the heterozygous state. In the representative gel picture (Figure 4.1), the amplified PCR product for GMLQ975 showed resistant alleles with 150 bp and a susceptible allele of 280 bp which was in accordance with earlier studies (Pandey *et al*., 2017). Results based on gel electrophoresis could not clearly reveal polymorphism between the parents for GM2301 due to low resolution. Therefore, the PCR products were subjected to capillary electrophoresis where the clear polymorphism between the parents was reported. The electropherogram assay (Figure 4.2) showed the donor (ICGV 13193) and recipient (K 6) parents amplified with 235 bp (resistance) and 260 bp alleles (susceptible), respectively for GM2301 marker associated with rust resistance.

4.2 Introgression of alleles for high oleic acid trait into Kadiri 6 cultivar

To meet the market needs, groundnut kernel quality is equally important as the productivity traits and biotic stress resistance. Breeding high oleic groundnut varieties with the altered proportion of two fatty acids, oleic and linoleic acid is an important breeding objective. High oleic groundnut kernels are preferred by the food industry for shelf-life benefits and by consumers for health benefits. Hence, breeding for high oleic groundnut lines is an important priority of groundnut improvement programs worldwide.

The use of molecular markers helps in selecting the plants in early generations with target traits. For high oleic acid trait, in groundnut, the Cleaved Amplified Polymorphic Sequences (CAPS) and allele-specific markers were developed to differentiate mutant and wild-type alleles of *ahFAD2A* and *ahFAD2B* (Lopez and Burow, 2004 and Chen *et al*., 2010). These markers are successfully utilized in groundnut breeding programs across the globe to select mutant *ahFAD2A* and *ahFAD2B* alleles to develop high oleic groundnut lines (Chu *et al*., 2011., Mienie and Pretorius, 2013., Xiuzhen *et al*., 2016., Janila *et al*., 2016a and Bera *et al*., 2018).

Table 4.1. Allele sizes of foreground markers linked to QTLs governing LLS and rust resistance

Trait	Linked markers	Resistant allele (bp)	Susceptible allele (bp)
Rust	IPAHM103	154	130
Rust	GM2301	235	260
Rust	GMRQ786	200	-
LLS	GMLQ975	150, 280	280

Table 4.2. Allele sizes of the foreground markers linked to *ahFAD2* mutant alleles

Target allele	Linked markers	Mutant allele (bp)	Wild-type allele (bp)
ahFAD2A	F435-F	203	290
ahFAD2B	F435	190, 290	290

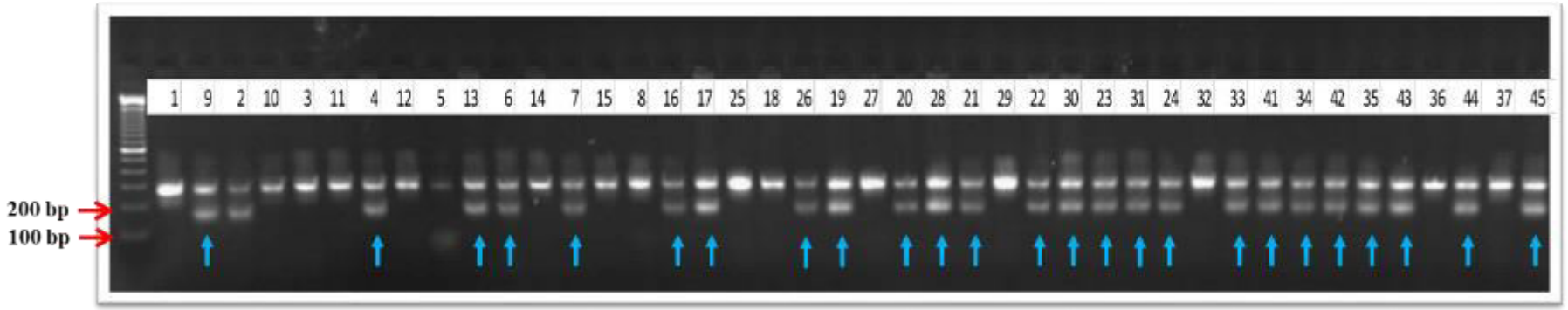

Figure 4.1 Confirmation of heterozygosity of F_1 plants derived from a cross between Kadiri 6 and ICGV 13193 using gene-specific marker GMLQ975 for LLS; Lanes; 1: Recipient (Kadiri 6), 9: Donor (ICGV 13193), 2-45: F_1 plants, with arrows indicated for true hybrids.

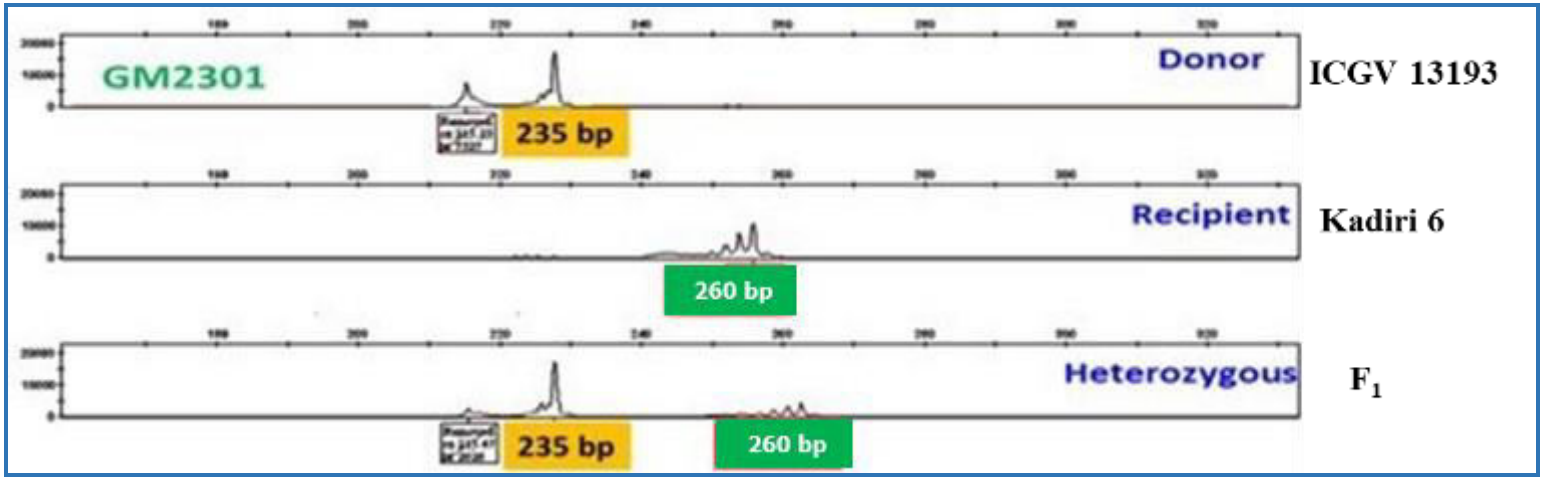

Figure 4.2 Electropherogram showing the heterozygous alleles amplified using SSR marker GM2301 for rust in a cross between Kadiri 6 and ICGV 13193; PCR products were separated with capillary electrophoresis.

In the present study, the advanced breeding line ICGV 15033 (ICGV 06420 × SunOleic 95R) developed at International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru (Janila *et al*., 2016a) was used as a donor parent to transfer high oleic trait into K 6. The pistillate parent K 6 was crossed with pollen parent, ICGV 15033 during rainy season 2016. A total of 229 F_1 seeds were obtained from this cross. The F_1 seeds were planted in the greenhouse during post-rainy season 2016-17 and 210 seedlings were screened for hybridity test using allele-specific makers. The gel pictures were analyzed for the presence of the target mutant alleles of *ahFAD2A and ahFAD2B* for the high oleic acid trait. Out of 210 plants screened, 151 (72%) plants showed the donor parent type heterozygous alleles with a size of 190 bp and 290 bp. The primer combination, F435-F, F435, and F435IC-R was used as internal control to confirm the amplification of 290 bp fragment for wild-type allele (Table 4.2 and Figure 4.3).

4.3 Combining QTLs for late leaf spot, rust resistance and mutant *ahFAD2* alleles in Kadiri 6 cultivar

In the present investigation, Marker Assisted Selection (MAS) was undertaken to combine QTLs for LLS and rust resistance and mutant *ahFAD2* alleles for high oleic traits. Molecular markers tightly linked to resistance genes and high oleic trait have facilitated their pyramiding. The positive F_1s obtained from two separate single crosses (K 6 × ICGV 15033; K 6 × ICGV 13193) were intercrossed during post-rainy 2016-17 to combine LLS, rust resistance and mutant *ahFAD2* alleles (Table 4.4). A total of 520 seeds were harvested from this intercross.

Gel-based SSRs, allele-specific markers are accurate and expedite many breeding programs however, it holds certain limitations such as tedious and laborious. Furthermore, unclear banding pattern when genotyped on polyacrylamide gel electrophoresis (PAGE) and electropherogram peak pattern when analyzed on the capillary electrophoresis demands a repetition of these experiments (Pandey *et al*., 2017). The other onerous issue is the availability of time to genotype the segregating breeding populations to select the true hybrid F_1 plants for making backcrosses, which gives only 10- 15 days of time window before flowering ends. This affair hinders the large scale adoption and deployment of these linked markers in a breeding program.

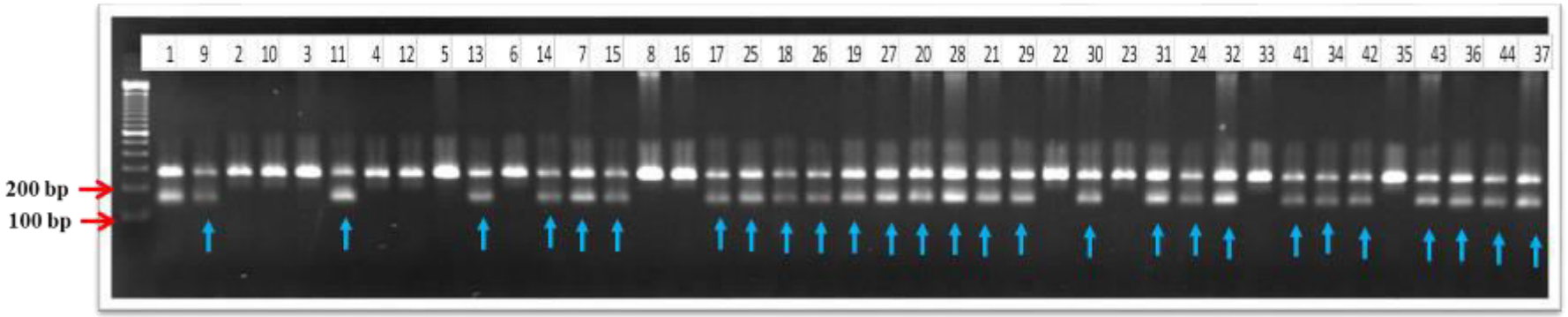

Figure 4.3 Confirmation of heterozygosity of F_1 plants derived from a cross between Kadiri 6 and ICGV 15033 with allele-specific marker F435-INS linked to mutant allele *ahFAD2B*; Lanes- 1: Donor (ICGV 15033), 2: Recipient (Kadiri 6), 3-44: F_1 plants, with arrows indicated for true hybrids.

The development of SNP markers and array-based genotyping protocols, along with improvements in laboratory automation has increased the capacity of MAS, enabling high-throughput sample screening, and thus saves the time required to genotype the large set of the population (Pandey *et al*., 2017 and Desmae *et al*., 2018). Recently, QTL sequencing (QTL-seq) strategy was deployed to map genomic regions associated with resistance to LLS and rust in groundnut. This study developed 26 diagnostic SNPs derived from earlier mapped QTLs on linkage group A03 for LLS and rust resistance (Pandey *et al*., 2017).

4.3.1 Kompetitive Allele Specific PCR (KASP) based SNP assay for zygosity test

Based on identified mutations in *ahFAD2A* and *ahFAD2B* alleles, the diagnostic SNPs were constructed at ICRISAT and shared with Intertek Pvt Ltd, Hyderabad for initial testing and validation in Kompetitive allele-specific PCR (KASP). The initial standardization and wet-lab validation of KASP assay come up with a diagnostic SNP marker for high oleic acid, LLS and rust resistance traits. Five SNP markers on linkage group (LG) A03 associated with LLS and rust resistance and one SNP marker on LG B09 associated with high oleic trait was utilized for genotyping. Small leaf disc (2 mm) samples are collected from individual plants of a breeding population and genotyped on KASP based SNP genotyping platform.

In KASP genotyping assay, five SNPs for LLS and rust *viz*., snpAH0015, snpAH0017, snpAH0018, snpAH0021 and snpAH0026 showed a base combination of T:T, A:A, G:G, C:C and G:G for resistant type alleles. Another snpAH002 showed A:A and A:- respectively for homozygous and heterozygous allele call for the *ahFAD2B* allele (Figure 4.4). The SNPs developed for *ahFAD2A* were uncallable.

In the summer season of 2017, 415 seedlings from $F_1 \times F_1$ cross were raised and genotyped using KASP assay to identify plants with high oleic acid, LLS and rust resistance alleles. In the hybridity test, 44 F_1 plants were found positive with 11% hybridity output. These positive plants showed heterozygote alleles for SNPs for all three traits i.e. LLS and rust resistance, and *ahFAD2B* (for high oleic acid trait). The positive F_1 plants (44) were used as a pollen parent to make the first backcross with the recurrent parent K 6 and a total of 420 BC_1F_1 seeds were harvested. Of these 420 BC_1F_1 seeds, a total of 380 seedlings were raised in rainy 2017.

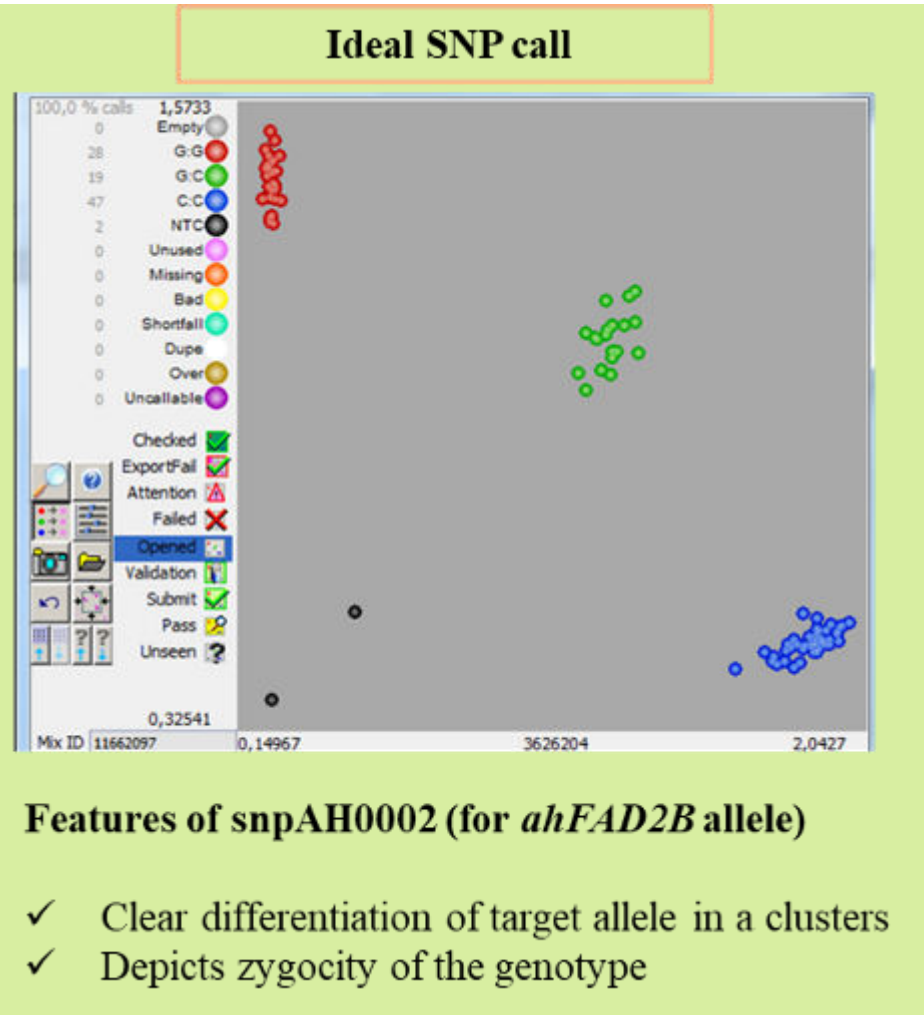

Figure 4.4 Kompetitive Allele Specific PCR (KASP) graphs of SNPs genotyping with snpAH0002 for *ahFAD2B* allele; (The scatter plot with axes x and y represents allelic discrimination. Red, green and blue dots represent the homozygous, heterozygous and wild type alleles, respectively).

The hybridity test was carried out with six SNPs viz., snpAH0015, snpAH0017, snpAH0018, snpAH0021, snpAH0026 and snpAH002 which resulted in the identification of 41 positive BC_1F_1 plants with heterozygous target alleles for LLS, rust, and *ahFAD2B*. These positive BC_1F_1 plants were selfed and 396 seeds were collected.

KASP assay greatly facilitates the plant breeding program due to accuracy, quick assay and less resource demanding. For marker-assisted selection, use of a limited number of KASP markers over many samples has advantages of cost, simplicity, and speed (Chopra *et al*., 2018). In earlier reports, KASP assay has been successfully deployed in groundnut breeding program for the traits such as rust resistance (Leal-Bertioli *et al*., 2015), nematode resistance (Chu *et al*., 2016), detection of mutant *ahFAD2* alleles for oil quality analysis (Chopra *et al*., 2015 and Zhao *et al*., 2017), bacterial wilt resistance (Zhao *et al*., 2016), LLS resistance (Clevenger *et al*., 2018), plant architectural traits (Chopra *et al*., 2018), early leaf spot and LLS, and TSWV (Agarwal *et al*., 2018).

The marker-assisted gene pyramiding approach has been successfully utilized in many crop plants to improve an existing elite cultivar through introgression of a number of genes from more than one donor (Tyagi *et al*., 2014). The normal breeding of an elite groundnut cultivar typically takes 6-8 of breeding cycles and another 3-5 years of testing for release or commercialization. The development useful molecular markers, new and improved phenotyping methods, and efficient breeding strategies assist the breeders to develop new groundnut cultivars with enhance rate of genetic gain for the target traits, resistance to LLS and rust and high oleic traits in this specific case.

Backcrossing is the preferred strategy for transferring a few useful genes from a donor parent into an adapted cultivar or elite breeding line (Allard, 1960). A scheme of the simultaneous crossing of a recurrent parent with many donor parents and intercrossing F_1s from different crosses followed by backcrossing with a recurrent parent is a promising method of gene pyramiding. This scheme was utilized in many crops to pyramid bacterial leaf blight and lepidopteran insect pest tolerance in rice (Jiang *et al*., 2004), bacterial leaf blight in rice (Shanti *et al*., 2010), stripe rust and leaf resistance genes in wheat (Santra *et al*., 2006 and Yadawad *et al*., 2017), leaf rust, net blotch and spot blotch resistance in barley (Hickey et *al*., 2017). This is one of the most reliable gene pyramiding method as it reduces the time period to pyramid genes/ QTLs and assures the fixation of genes/ QTLs (Joshi and Nayak, 2010).

In the MABC program, one to four backcrosses is needed to recover the yield and quality characteristics of the recurrent parent (Sundaram *et al*., 2012 and Kolekar *et al*., 2017). More rounds of backcrossing have advantages when the adaptation of donor parents (wild relatives/ germplasm/ landraces) is much poorer than that of the adapted parents. Even though repeated cycles of backcrossing are useful to increase the proportion of recurrent parent genome, each additional backcrossing increases the probability that target genes get reduced (Wang *et al*., 2009) especially when the progenies are introgressed with QTLs and resistance is controlled by a combination of large and small effect QTLs. This phenomenon was observed by Kolekar *et al*. (2017) where recovery of a number of resistant progeny for rust were more over the LLS. This could be due to the simple genetic control of rust resistance over the more complex genetic control of LLS resistance. In such backcross breeding program, one or two backcrosses are enough to introgress trait/s of interest. A strategy of single backcrossing has been successfully used in wheat improvement at CIMMYT to introgress rust resistant genes from donor parents to elite adapted wheat cultivars (Wang *et al*., 2009 and Yadawad *et al*., 2017), leaf rust, net blotch and spot blotch resistance in barley (Hickey et *al*., 2017). In groundnut, Kolekar *et al*. (2017) attempted single backcrossing to improve TMV 2 for LLS and rust resistance. The background genome recovery in BC_1F_1 generation can range between 76.83- 84.14 %, averaging 79.88 % as reported in maize (Yang *et al*., 2013). These studies indicated that crossing scheme with one to two rounds of backcross has advantages in retaining or improving the adaptation of the recurrent parents, and at the same time transferring most of the desired genes from the donor parent.

In the present study, both the donor parents, ICVGs 15033 and 13193 are advanced breeding lines possessing good agronomic characters (Janila *et al*., 2015a and 2015b). We successfully transferred QTLs for resistance to LLS and rust into the K 6 genetic background within a two-year period using a marker-assisted selection approach coupled with the use of high-throughput genotyping and rapid generation advancement. Background selection for large numbers of plants with molecular markers/ array based genotyping at each backcross generation would be an expensive task and logistically challenging.

Whereas, MAS combined with multi-trait screening assays and selection of recurrent parental features based on phenotype and assessment of background genome recovery in later generations through marker analysis can accelerate backcross breeding programs and make them cost-effective and resource efficient (Pandey *et al*., 2013).

Therefore, in the present study marker-assisted foreground selection coupled with phenotypic screening for disease resistance, field evaluation for morpho-agronomic traits and kernel quality evaluation by near-infrared reflectance spectroscopy (NIRS) was used.

In summer 2018, 343 BC_1F_2 seedlings were raised in the field and screened for zygosity using KASP based SNP markers. The assay revealed 16 plants with homozygous alleles for LLS, rust, and *ahFAD2B* (Table 4.3 and 4.4). KASP platform enabled us to genotype many samples without the need to design specific multiplex sets. The genotyping data turnaround time for the genotype-driven breeding cycles was one of the most critical components. All the tasks related to genotyping (leaf sampling, DNA extraction, genotyping, and data analyses) saved time due to the deployment of KASP assay and the genotypic results were ready in about 20 days. This process helped in the pre-flowering selection of plants for crossing and rapid generation advancement.

4.4 Phenotypic evaluation of backcrossed lines for late leaf spot and rust resistance

The backcrossed progenies of BC_1F_2s were evaluated for LLS and rust response under controlled conditions by using a detached leaf assay. Detached leaf assay was proposed by Foster *et al*. (1980) as a rapid and simple method for screening groundnut genotypes for leafspot resistance. Other methods such as Hoagland's solution (Foster *et al*., 1980), whole seedling assay planted in pots (Dwivedi *et al*., 2002) and sand culture (Janila *et al*., 2013a) were used in previous studies to screen genotypes against LLS resistance in groundnut. In the present study, the inclusion of sand: vermiculite culture (1:1 v/v) composition enabled maintenance of optimum moisture in the culture and relative humidity (≥85%) with minimum contamination of saprophytes and secondary infection. The detached leaf assay offers an advantage for off-season screening against LLS and rust disease. Overall, a reasonable degree of environmental control was needed to achieve the right balance between withstand of detached leaflet and conditions ideal for both disease infection and development.

Table 4.3. Kompetitive allele-specific PCR (KASP) assay identified the homozygous alleles for late leaf spot and rust resistance and high oleic acid traits in K6 derived BC_1F_2 progenies

S. No.	Genotypes	HOA	Late leaf spot and rust				
		snpAH002	snpAH0015	snpAH0017	snpAH0018	snpAH0021	snpAH0026
1	ICGV 13193 (donor parent for FDR)	-:-	T:T	A:A	G:G	C:C	G:G
2	ICGV 15033 (donor parent for high oleic traits)	A:A	T:T	A:A	G:G	C:C	G:G
3	Kadiri 6 (recipient parent)	-:-	A:A	C:C	A:A	G:G	C:C
4	BC_1F_2-4	A:A	T:T	A:A	G:G	C:C	G:G
5	BC_1F_2-5	A:A	T:T	A:A	G:G	C:C	G:G
6	BC_1F_2-34	A:A	T:T	A:A	G:G	C:C	G:G
7	BC_1F_2-76	A:A	T:T	A:A	G:G	C:C	G:G
8	BC_1F_2-120	A:A	T:T	A:A	G:G	C:C	G:G
9	BC_1F_2-144	A:A	T:T	A:A	G:G	C:C	G:G
10	BC_1F_2-186	A:A	T:T	A:A	G:G	C:C	G:G
11	BC_1F_2-214	A:A	T:T	A:A	G:G	C:C	G:G
12	BC_1F_2-216	A:A	T:T	A:A	G:G	C:C	G:G
13	BC_1F_2-225	A:A	T:T	A:A	G:G	C:C	G:G
14	BC_1F_2-249	A:A	T:T	A:A	G:G	C:C	G:G
15	BC_1F_2-269	A:A	T:T	A:A	G:G	C:C	G:G
16	BC_1F_2-278	A:A	T:T	A:A	G:G	C:C	G:G
17	BC_1F_2-296	A:A	T:T	A:A	G:G	C:C	G:G
18	BC_1F_2-311	A:A	T:T	A:A	G:G	C:C	G:G
19	BC_1F_2-327	A:A	T:T	A:A	G:G	C:C	G:G

HOA: High oleic acid trait; -:- null allele

Table 4.4. Summary of Kompetitive allele-specific PCR (KASP) assay for target traits

SNP combinations	Total genotypes	Rust score *	LLS score †	Oleic acid (%) ¥
All six target SNPs	16	3-4	3-4	61.2-83.4
High oleic SNP	71	4-7	4-7	60.4-83.4
LLS and rust resistance associated SNPs	45	3-4	3-5	40.6-55.2
Null allele for LLS and rust resistance and high oleic trait	211	5-8	5-8	38.4-52.6

*- Range of rust score at 35 days after inoculation (DAI), †-range of LLS score at 35 DAI,

¥- range oleic acid content in selected genotypes based on presence of SNPs for target traits

The results on the screening of fully expanded excised quadrifoliate leaves provided a clear differentiation between susceptible, resistant and moderately resistant cultivars (Figure 4.5) based on artificial inoculations with spore concentration higher than 30,000 conidia ml^{-1}. Spore suspension of ≥ 20,000 conidia ml^{-1} is sufficient to build the proper inoculum load to screen groundnut test entries with LLS and rust disease (Motagi, 2001). As disease severity in the susceptible cultivar increased with duration, the difference between susceptible and resistant cultivars became more evident. The results of phenotypic evaluation for LLS and rust resistance of 16 BC_1F_2 progenies are presented below.

4.4.1 Late leaf spot

The components of resistance *viz.*, incubation period, sporulation period, number of lesions per leaflet, lesion diameter, percent leaf area damaged and LLS score was considered to access the resistance of progenies against LLS. The data recorded on these components of resistance from individuals of BC_1F_2 along with donor and recipient parents and resistant and susceptible check is presented in Table 4.5.

The range of incubation period (IP) was 9.5 to 12.8 days after inoculation (DAI) in backcross progenies with highest IP in BC_1F_2-327 (12.8 DAI), while recurrent parent K 6 showed IP of 7.0 DAI. The sporulation duration was ranged from 21.5- 24.7 DAI. The BC_1F_2s-144, 186 and 269 showed longer sporulation period (24.5 to 24.7 DAI) as compared to recipient parent K 6 (15.0 DAI) and susceptible check, TMV 2 (13.5 DAI).

The mean number of lesion per leaflet was ranged from 19.5 to 78.5 at 35 DAI. The minimum number of lesions were observed in progenies BC_1F_2-4 (19.5), BC_1F_2-5 (27.0), BC_1F_2-120 (38.0), and BC_1F_2-34 (44.0). Recurrent parent, K 6 recorded 140 lesions per leaflet, whereas donor parent ICGV 13193 had 32.5 lesions per leaflet. The lesion diameter in the 16 BC_1F_2 individuals ranged from 2.1 to 4.6 mm as compared to K 6 (5.7 mm) and ICGV 13193 (1.6 mm). The lower lesion diameter was recorded in BC_1F_2-76 (2.1 mm), BC_1F_2-296 (2.2 mm), and BC_1F_2-225 (2.3 mm). The percent leaf area damaged by LLS was lower in BC_1F_2-296 (15.5%) and BC_1F_2-216 (19.5%). The parental lines K 6 and ICGV 13193 were recorded 61.0% and 21.0% leaf area damaged by LLS respectively. The score of LLS was recorded with a range of 3.0 to 5.0 in progenies.

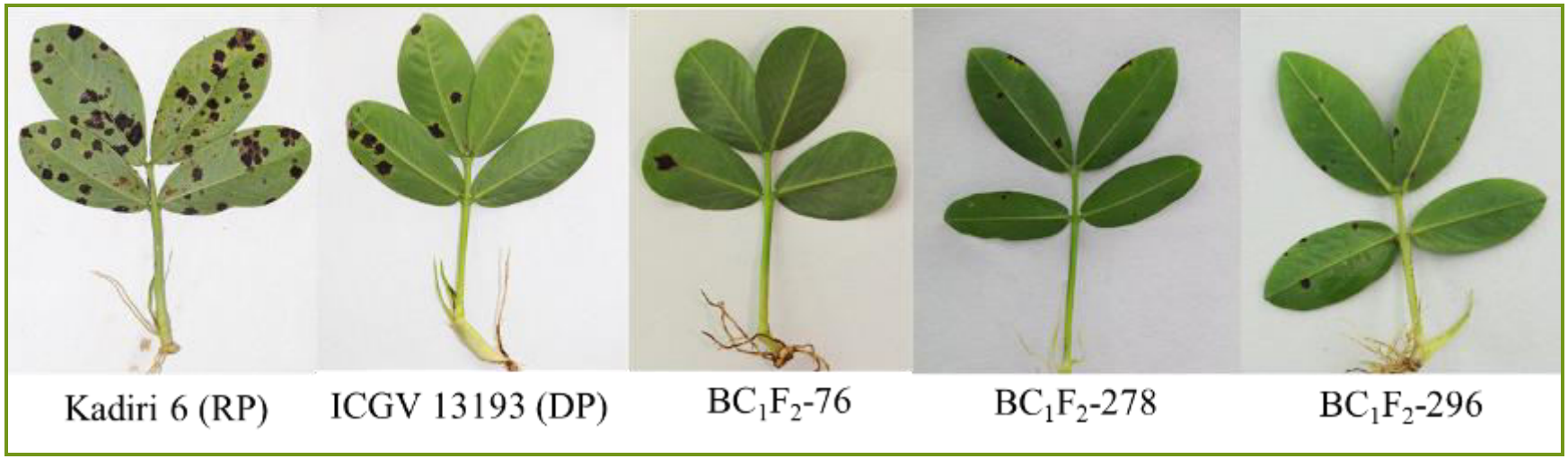

Figure 4.5 Late leaf spot and rust reaction of the recurrent parent (Kadiri 6), donor parent (ICGV 13193) and its backcross progenies at 35 DAI

Table 4.5. Components of resistance and disease score of Kadiri 6 derived backcross progenies (BC_1F_2) against late leaf spot

S. No.	Genotypes	IP (DAI)	SP (DAI)	LPL (45 DAI)	LD (mm)	% LADL	LLS score (35 DAI)
1.	BC_1F_2-4	11.5	23.5	19.5	3.3	21.0	3.5
2.	BC_1F_2-5	11.5	22.9	27.0	3.5	24.5	3.5
3.	BC_1F_2- 34	10.0	21.9	44.0	4.5	24.0	3.5
4.	BC_1F_2-76	10.6	22.7	74.0	2.1	37.7	3.0
5.	BC_1F_2-120	12.0	23.5	38.0	2.8	24.5	5.0
6.	BC_1F_2-144	11.5	24.5	72.5	3.5	22.5	3.5
7.	BC_1F_2-186	9.9	24.5	62.0	2.5	22.7	3.5
8.	BC_1F_2-214	9.5	22.5	61.5	3.1	24.0	4.0
9.	BC_1F_2-216	11.5	23.4	76.5	3.4	19.5	3.0
10.	BC_1F_2-225	11.7	22.7	78.5	2.3	32.5	3.0
11.	BC_1F_2-249	12.6	23.5	68.6	3.4	21.0	3.5
12.	BC_1F_2-269	11.6	24.7	63.5	4.6	21.0	4.0
13.	BC_1F_2-278	9.9	23.5	61.0	2.5	22.5	3.5
14.	BC_1F_2-296	10.8	21.5	61.5	2.2	15.5	3.5
15.	BC_1F_2-311	11.9	22.5	55.0	3.1	25.5	3.0
16.	BC_1F_2-327	12.8	21.5	44.5	4.2	23.0	3.5
17.	ICGV 13193 (Donor parent for FDR)	11.7	23.5	32.5	1.6	21.0	3.0
18.	ICGV 15033 (Donor parent for high oleic trait)	9.9	18.5	93.0	4.3	33.5	5.0
19.	Kadiri 6 (Recipient parent)	7.0	15.5	140.0	5.7	61.0	7.5
20.	GPBD 4 (Resistant check)	15.6	25.7	13.5	1.2	20.7	3.0
21.	TMV 2 (Susceptible check)	7.1	13.5	153.0	6.0	64.5	8.5
	SEm ±	1.3	0.4	2.7	0.3	1.2	0.4
	LSD (5%)	1.1	1.3	8.0	0.8	3.5	1.2

IP: Incubation Period (DAI), SP: Sporulation Period (DAI), LPL: Lesion Per Leaflet, LD: Lesion Diameter (mm), % LADL: percent Leaf Area Damaged by LLS, LLS: Late Leaf Spot, DAI: Days After Inoculation, SEm: Standard Error of the Mean, LSD: Least Significant Difference

The progenies *viz*., BC_1F_2s-76, 216, 225 and 311 were found to be resistant for LLS with a score of 3.0; whereas K 6 (RP), ICGV 13193 (DP for LLS and rust) and ICGV 15033 (DP for high oleic trait) had LLS scores of 7.5, 3.0 and 5.0 respectively at 35 DAI. Walls *et al*. (1985) suggested that latent period, lesion area and amount of sporulation could be used as measurements of resistance to predict the disease reaction of a line in the field. Dwivedi *et al*. (2002) concluded that the resistance to LLS is due to longer incubation and lesser lesions per leaf, smaller lesion diameter, lower sporulation index, and lesser leaf area damage and disease score. The disease reaction of detached leaflets inoculated with artificial inoculum, determined using both disease incidence and severity values were similar to the reaction of the intact plant used in their study. These investigations suggest that detached leaf assay gives the concrete and conclusive evidences for the LLS and rust resistance in test genotypes.

4.4.2 Rust

The components of resistance for rust *viz*., incubation period, sporulation period, number of pustules per leaflet, percent leaf area damaged by rust and rust score were considered to assess the rust resistance of the 16 backcrossed progenies. The data of each individual of BC_1F_2 for these component traits were compared with donor and recipient parent and data is shown in Table 4.6. The parental lines K 6 and ICGV 13193 recorded incubation period of 9.5 and 14 days after inoculation (DAI), respectively. In contrast, the incubation period for rust disease among BC_1F_2s was ranged from 10.0 to 13.5 DAI. The higher incubation period of 13.5 DAI was observed in the BC_1F_2s-120 and 186. The sporulation period was ranged from 15.5-19.5 DAI. The four progenies (BC_1F_2s-5, 120, 225 and 249) were observed with the highest sporulation period of 19.5 DAI. The sporulation period in K 6 was 12 days and in ICGV 13193 it was 19 DAI. The number of rust pustule per leaflet was ranged from 19.5-68.5. The progenies *viz.*, BC_1F_2-225 (19.5) and BC_1F_2-5 (21) were observed with lower rust pustules per leaflet. The parental lines K 6 and ICGV 13193 were measured with 98.5 and 19.5 pustules per leaflet respectively. The percent leaf area damaged by rust pustules was measured between 9.0 to 24.5 %. The lower percent leaf area damaged by rust pustules was observed in BC_1F_2-249 (9%), BC_1F_2-144 (9.5%), BC_1F_2-269 (11.0%). The parental lines K 6 and ICGV 13193 were estimated with 25.0% and 8.5% leaf area damaged by rust pustules respectively. The rust disease was scored with a range of 3.0 to 4.0 in BC_1F_2 progenies, whereas K 6 (RP), ICGV 13193 (DP for LLS and rust) and ICGV 15033 (DP for high oleic trait) had rust scores of 7.0, 3.5 and 4.0 at 35 DAI, respectively (Figure 4.6).

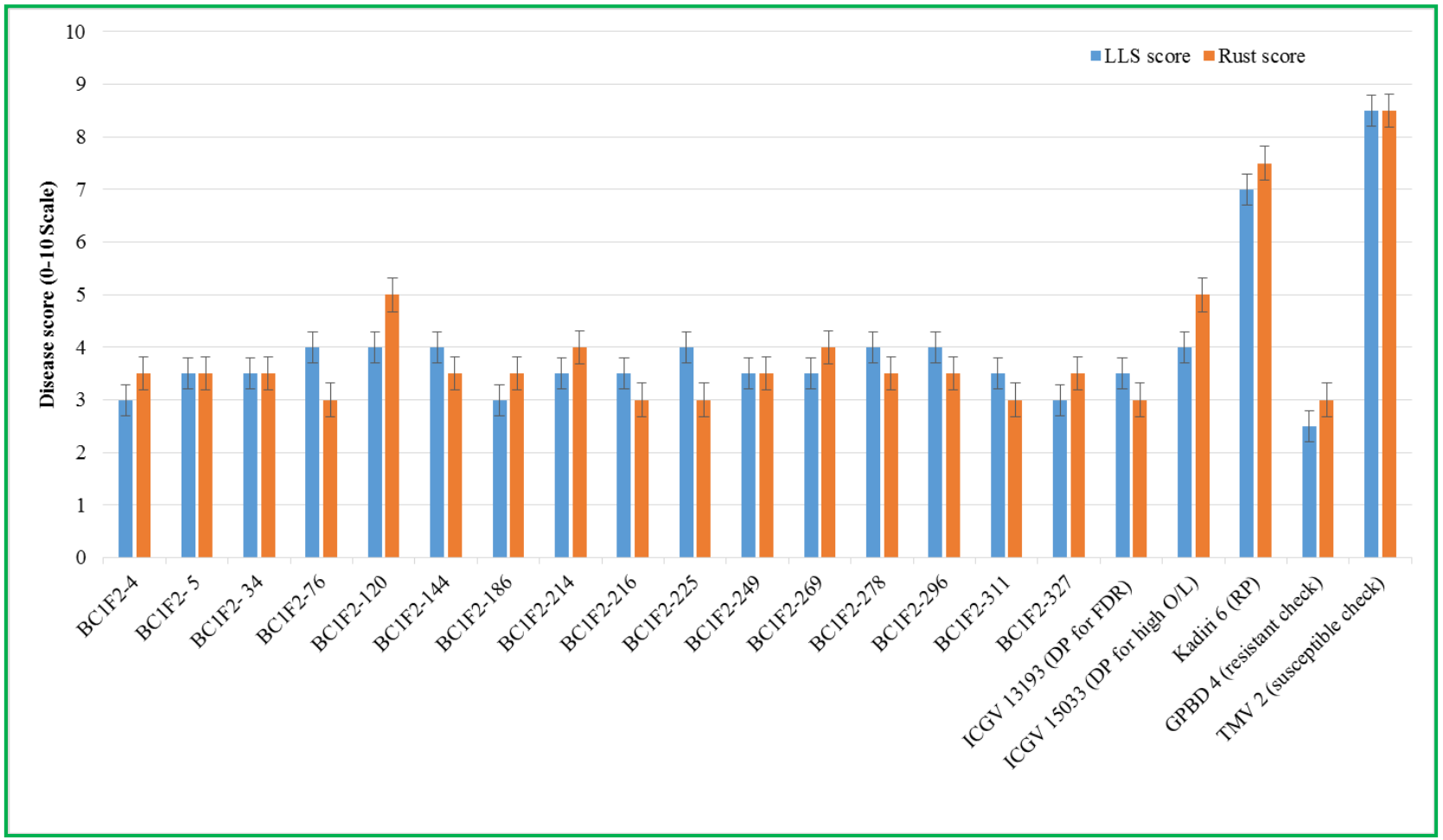

Figure 4.6 Late leaf spot and rust scores of the recurrent parent (Kadiri 6), donor parents (ICGV 13193 and ICGV 15033) and its backcross progenies

Table 4.6. Components of resistance and disease score of Kadiri 6 derived backcross progenies (BC_1F_2) against rust

S. No.	Genotypes	IP (DAI)	SP (DAI)	PPL	%LADR	Rust score (35 DAI)
1.	BC_1F_2-4	12.5	18.5	23.0	15.5	3.0
2.	BC_1F_2- 5	13.5	19.5	21.0	13.0	3.5
3.	BC_1F_2- 34	11.5	17.5	21.5	19.0	3.5
4.	BC_1F_2-76	11.0	17.0	66.5	24.5	4.0
5.	BC_1F_2-120	13.5	19.5	25.0	11.5	4.0
6.	BC_1F_2-144	12.5	17.5	22.5	9.5	4.0
7.	BC_1F_2-186	13.5	18.0	25.0	21.0	3.0
8.	BC_1F_2-214	12.5	16.0	30.5	18.5	3.5
9.	BC_1F_2-216	10.0	17.5	26.0	14.5	3.5
10.	BC_1F_2-225	12.5	19.5	19.5	12.5	4.0
11.	BC_1F_2-249	11.5	19.5	21.5	9.0	3.5
12.	BC_1F_2-269	10.5	16.0	37.5	11.0	3.5
13.	BC_1F_2-278	12.0	18.0	68.5	20.5	4.0
14.	BC_1F_2-296	11.5	15.5	52.0	11.5	4.0
15.	BC_1F_2-311	12.5	18.0	29.5	12.5	3.5
16.	BC_1F_2-327	12.5	16.5	24.0	13.5	3.0
17.	ICGV 13193 (Donor parent for FDR)	14.0	19.0	19.5	8.5	3.5
18.	ICGV 15033 (Donor parent for high oleic trait)	12.5	15.5	22.5	10.5	4.0
19.	Kadiri 6 (Recurrent parent)	9.5	12.0	98.5	25.0	7.0
20.	GPBD 4 (Resistant check)	16.5	22.5	12.5	7.5	2.5
21.	TMV 2 (Susceptible check)	8.5	10.5	122.5	40.0	8.5
	SEm ±	0.4	1.1	2.3	1.3	0.4
	LSD (5%)	1.3	3.2	6.8	3.8	1.2

IP: Incubation Period (DAI), SP: Sporulation Period (DAI), PPL: Pustule Per Leaf, %LADR: percent Leaf Area Damaged by Rust, DAI: Days After Inoculation, SEm: Standard Error of the Mean, LSD: Least Significant Difference

The components of rust resistance such as longer latent period, fewer pustules per leaf, smaller pustule size, lower sporulation period, and lesser leaf area damage should be taken into consideration during selection of resistant genotypes (Mehan *et al*., 1994). Similarly, Dwivedi *et al*. (2002) observed that the resistance to rust is due to longer incubation and latent periods, fewer pustules per leaflet, smaller pustule diameter, lower sporulation period, lesser leaf area damage and lesser disease score. The incubation period was the most frequent component of resistance involved in rust-resistant genotypes followed by pustule diameter, sporulation index, and percent leaf area damaged in their findings.

4.5 Evaluation of backcrossed lines for morpho-agronomic and kernel quality traits

The backcrossed lines screened under a number of stress conditions can be used to select lines that outperformed the elite recurrent parent (Collard and Mackill, 2007). The genotype of the best performing introgression lines can be compared with the recurrent parent to look for a significant association between specific donor introgressions and performance in the population of lines that survived under the disease screening assay (Yadawad *et al*., 2017). This approach allows identification of more than one introgressions from the donor and easy identification of small effect QTL with accuracy.

With MAS, individual plants can be selected based on their genotype. For most traits, homozygous and heterozygous plants cannot be distinguished by conventional phenotypic screening. These advantages can be exploited in single plant selection to accelerate the breeding process (Ribaut and Hoisington, 1998 and Morris *et al*., 2003). Target genotypes can be more effectively selected, which enables certain traits to be 'fast-tracked', resulting in quicker line development. MAS allows selection in off-season nurseries making it more cost effective to grow more generations per year (Ribaut and Hoisington, 1998). In MAS, the total number of lines that need to be tested can be reduced. Since many lines can be discarded after MAS early in a breeding scheme, this permits more efficient use of glasshouse and/or field space (Collard and Mackill, 2007). In MAS, phenotypic selections in an early generation for positive introgressed lines eliminates the chances of deleterious alleles, the likelihood of detecting favorable introgressions from the donor parents is increased.

In the present study, the aim of the field screening was to identify individual plants resembling recurrent parent K 6 and identify progenies featuring on par to superior for morpho-agronomic characters. The recipient parent has Spanish bunch-type growth habit with the features *viz*., 100 to 105 days of maturity, 70% shelling percentage, 39 g mass of sound mature kernels, and 50% oil content. Groundnut descriptor jointly published by the International Board for Plant Genetic Resources (IBPGR) and ICRISAT (1992) was referred to record morpho-agronomic features of K 6 and individuals of BC_1F_2.

4.5.1 Morpho-agronomic traits

A set of 343 BC_1F_2 progenies were raised during the summer, 2018 (March to June) in the field condition to evaluate for morpho-agronomic features. A total of 16 plants were found homozygous for LLS, rust and high oleic acid traits. The morpho-agronomic performance of 16 backcross progenies of K 6 (BC_1F_2) is presented in Table 4.7.

Days to 50% flowering was recorded with a range from 31 to 38 days (Table 4.7). The average days to 50% flowering in the population were 34.15 days. The recurrent parent K 6 took 35 days for 50% flowering. Earliness helps to avoid the late-season biotic and abiotic stresses and also makes the crop fit for intensive cropping system which is important for profitable and sustainable farming (Poehlman and Sleper, 1995).

The plant height of the main axis ranged from 17.0 cm (BC_1F_2-186) to 38.4 cm (BC_1F_2-214). The mean plant height was recorded with 26.29 cm. The plant height of recurrent parent, K 6 was 34.6 cm. Nigam and Aruna (2008) indicated that short plant stature and accumulation of maximum numbers of early flowers are important traits to develop short duration groundnut cultivars.

The number of mature pods per plant ranged from 24 to 58 with a mean of 38.5. The progeny, BC_1F_2-269 recorded 58 pods per plant followed by BC_1F_2-120 and BC_1F_2-144 with 55 and 54 pods respectively. The recurrent parent K 6 recorded with 30 pods per plant. The genotypes having a higher number of pods per plant offer an opportunity for improving seed yield in groundnut (Luz *et al.*, 2011).

Table 4.7. Morpho-agronomic and pod traits of backcross progenies of K 6 (BC_1F_2) evaluated during the summer of 2018 at ICRISAT, Patancheru

S. No.	Genotypes	DFF	PH (cm)	NPP	PY/P (g)	SY/P (g)	SH (%)	PL (mm)	PW (mm)	PCN	PR	PB	PRG
1.	BC_1F_2-4	32.0	29.5	29.0	20.7	14.6	72.0	25.1	10.7	S	S	S	M
2.	BC_1F_2-5	32.0	18.5	30.0	28.7	21.5	74.9	25.8	10.6	S	S	S	A
3.	BC_1F_2-34	35.0	27.5	30.0	24.0	18.8	78.3	25.9	11.9	M	M	M	S
4.	BC_1F_2-76	35.0	21.0	38.0	28.1	20.5	73.0	18.8	8.7	S	S	S	S
5.	BC_1F_2-120	34.0	24.5	55.0	33.8	25.1	74.3	19.5	9.3	S	S	S	A
6.	BC_1F_2-144	31.0	19.0	54.0	38.0	24.8	65.3	26.4	11.7	S	S	S	S
7.	BC_1F_2-186	32.0	17.0	24.0	19.6	15.4	78.6	24.9	10.5	S	S	S	A
8.	BC_1F_2-214	34.0	38.4	27.0	17.2	11.8	68.6	27.4	8.9	S	M	S	M
9.	BC_1F_2-216	35.0	22.0	38.0	29.1	21.9	74.0	19.8	11.7	S	M	S	S
10.	BC_1F_2-225	31.0	29.2	40.0	31.4	24.2	70.0	20.5	10.8	S	S	S	S
11.	BC_1F_2-249	32.0	30.4	31.0	25.9	22.0	68.3	21.4	9.9	S	S	S	S
12.	BC_1F_2-269	38.0	18.0	58.0	40.8	32.2	78.9	23.0	10.1	S	S	S	A
13.	BC_1F_2-278	34.0	29.0	45.0	39.7	29.2	73.6	20.7	9.1	S	S	M	A
14.	BC_1F_2-296	36.0	32.5	28.0	21.5	15.7	73.0	25.1	10.5	S	S	S	A
15.	BC_1F_2-311	32.0	31.2	42.0	32.2	23.2	72.0	25.5	9.8	S	S	S	A
16.	BC_1F_2-327	34.0	33.0	30.0	30.2	20.8	68.9	27.4	8.9	M	S	S	S
17.	ICGV 13193 (DP for FDR)	37.0	29.5	41.0	32.7	28.2	74.0	21.4	11.0	S	S	S	S
18.	ICGV 15033 (DP for high O/L)	36.0	30.0	33.0	25.3	19.3	76.3	20.5	9.8	S	S	S	A
19.	Kadiri 6 (recurrent parent)	35.0	34.6	30.0	26.8	19.8	73.9	24.5	11.5	M	M	P	M
	Mean	34.0	26.2	38.5	29.4	21.3	72.4	23.9	9.9	-	-	-	-
	Range	31.0-38.0	17.0-38.4	24.0-58.0	17.2-40.8	11.8-32.2	65.3-78.9	18.8-27.4	8.7-11.9	-	-	-	-

DFF: Days to 50% Flowering, PH: Plant Height (cm), NPP: Number of Pods per Plant, PY/P: Pod Yield per Plant (g), SY/P: Seed Yield per Plant (g), SH (%): Shelling outturn (%), PL: Pod Length (mm); PW: Pod Width (mm), PCN: Pod Constriction, PR: Pod Reticulation, PB: Pod Beak, PRG: Pod Ridges, S: Slight, M: Medium, P: Prominent, A: Absent

The weight of mature pods per plant ranged from 17.2 (BC_1F_2-214) to 40.8 g (BC_1F_2-269). The mean pod yield was 29.4 g. K 6 recorded a pod yield of 26.8 g/ plant. Pod yield was reported to be positively associated with the number and mass of mature pods per plant, the weight of mature kernels per plant, shelling outturn (Babariya and Dobariya, 2012). Thus, the selection was exercised for these associated traits simultaneously with the target trait. The mean seed weight per plant was 21.3 g with a range of 11.8 to 32.2 g per plant. The K 6 yielded 19.8 g of seeds per plant. The highest seed yield was recorded in progeny, BC_1F_2-269 with 32.2 g per plant.

Post-harvest traits of groundnut such as shelling out-turn, pod, and kernel related traits are equally important from an end user and industry point of view. In the present study, shelling out-turn was ranged from 65.3 to 78.9% with a mean of 72.4%. The higher shelling outturn of backcross lines was recorded in BC_1F_2-269 (78.9%) followed by BC_1F_2-186 (78.6%) and BC_1F_2-34 (78.3%). Shelling out-turn is an index of the percentage of kernels or seeds recovered after shelling (Dapaah *et al*., 2014) and it is one of the important selection criteria in groundnut (Anothai *et al*., 2008).

Pod and kernel-related traits in groundnut are important in overall appearance and adaptation and fetch higher market value and are traits for selection along with yield (Chen *et al*., 2016). In the present study, the mean length of mature pods was measured and it ranged from 18.8 to 27.4 mm. The minimum pod length was observed in BC_1F_2-76 (18.8 mm), while maximum pod length was observed in BC_1F_2s-214 and 327 (27.4 mm). The mean pod length was 23.91 mm in BC_1F_2 progenies. The pod length of K 6 was recorded with 24.5 mm. The width of mature pods was measured with a range of 8.7 mm (BC_1F_2-76) to 11.9 mm (BC_1F_2-34). Recurrent parent K 6 recorded a pod width of 11.5 mm.

Recipient parent featured pods with moderate constriction. A total of 14 progenies were observed with slight pod constriction. Two progenies (BC_1F_2-34 and BC_1F_2-327) observed with a moderate constriction like a recurrent parent. In general, most of the groundnut cultivars exhibit constriction to different degrees. Selection of pod with lesser constriction would help to improve K 6 for preferred pod features along with target traits. Pod constriction is an important character in the selection, as it affects the developing seed (Madhura *et al*., 2011). Pod reticulation was slight in 13 progenies and moderate in three progenies (BC_1F_2s-34, 214 and 216). Pod reticulation contributes to the clean harvest and has an impact on the quality and marketability of the produce.

In groundnut, the tip of the indehiscent fruit may end in an appearance called beak. Fourteen backcross progenies featured with slight beak whereas two progenies (BC_1F_2s-34 and 278) showed moderate beak. Selection of pods without beaks is an important feature as pods with beaks are susceptible to cracking in soil paving the way for the entry of *Aspergillus* and subsequent aflatoxin contamination. After drying, pod creates the crack towards the beak region which makes the kernels vulnerable for entry of storage pest and molds (Okello *et al*., 2010). The recipient parent K 6 had a prominent beak. Hence, priority was given to select the pods in progenies without beak and/or presence of slight beak.

The markings on pod referred to as pod ridges found to be moderate in two progenies, slight in seven progenies and absent in seven progenies. K6 was noted with moderate pod ridges. Janila *et al*. (2013b) suggested that reticulation, beak, and constriction of the pod are important considerations to satisfy farmers', processors', and market demands. Soil adheres to the pods with constriction and ridges during harvesting. Hence, pod characteristics *viz*., constriction, reticulation, beak, and ridges are undesirable traits. Bera and Das (1998) reported the monogenic inheritance pattern in pod beak and pod constriction. They concluded that pod non-beak and non-constricted pod type is dominant over the beak and constricted type pods. Hence, selection of pod features in early generation helps to identify the superior segregants. These segregants can possess superior pod features over its parents. In the current study, three such progenies (BC_1F_2s-76, 278 and 296) were obtained with superior pod shape over the recurrent parent.

4.5.2 Estimation of kernel quality traits

The oil, protein, and fatty acid contents are important criteria in trade, food, and seed industries of groundnut. Kernel and storage quality of groundnut is mainly governed by the relative proportion of three fatty acids *viz*., palmitic, oleic, and linoleic acid. Groundnut with a higher ratio of oleic to linoleic (O/L) acid is the preferred choice as it provides health benefits to the consumers by reducing low-density lipoprotein (LDL) levels in the blood, suppress tumorigenesis, and ameliorate inflammatory diseases (Alves *et al.,* 2014). High O/L groundnut kernels are preferred by the food processing industry as it retains shelf life longer by 8-10 times than normal groundnut (Wang, 2018).

Palmitic acid is known to cause an adverse effect on human health and found to increase the risk of developing cardiovascular diseases (Hu *et al*., 2001). Polyunsaturated fatty acids, especially higher linoleic acid is vulnerable to oxidation which leads to off-flavors (Braddock *et al*., 1995).

The traditional wet chemical methods for analysis of oil, protein and fatty acids content are accurate but operationally complex, time-consuming, destructive and expensive (Sundaram *et al*., 2009). In the present study, NIRS was utilized for simple, non-destructive, economic and fast screening of segregating progenies. The calibration equation used in prediction had high values of coefficient of determination in external validation (r^2) for oleic acid (r^2= 0.96), linoleic acid (r^2= 0.96), and moderate for oil (r^2= 0.89), protein (r^2= 0.83) and palmitic acid (r^2= 0.80). The kernel quality features *viz*., oil content, protein content, fatty acids composition, and oleic acid to the linoleic acid ratio (O/L ratio of the progenies are presented in Table 4.8.

4.5.2.1 Oil content (%)

The oil content in back cross progenies was ranged from 46.0% to 54.9% with an average of 50.8% (Table 4.9). The recurrent parent, K 6 recorded with 49.5% oil. The higher oil content (>50.0%) was observed in 11 progenies (BC_1F_2s-5, 76, 120, 144, 186, 214, 225, 249, 278, 296, 327). Oil content is an important parameter in groundnut which accounts for 44.0-56.0% of the dry seed weight (Janila *et al*., 2016c).

4.5.2.2 Fatty acids (%)

In the current study, three fatty acids (oleic acid, linoleic acid, and palmitic acid) were estimated using NIRS. The oleic acid ranged from 62.5% to 83.4% with a mean of 70.9%. The higher oleic acid was observed in three progenies BC_1F_2-76 (81.3%), BC_1F_2-278 (82.8%) and BC_1F_2-296 (83.4%). We observed 21.4 to 43.5% increase in oleic acid content among backcross progenies as compared to the recurrent parent (39.8%). A total of five selected progenies (BC_1F_2s-186, 214, 225, 249 and 311) showed above ≥70.0% oleic acid which is considered as moderate to high oleic lines. Progenies were estimated with a mean of 9.3% linoleic acid as compared to the recurrent parent, K 6 (37.4%). The linoleic acid levels recorded in progenies ranged from 25.4 to 43.5%. The oleic: linoleic acid (O/L) ratio was varied from 5.2 to 17.1. The higher O/L ratio (>10) was recorded in three progenies BC_1F_2s-76, 278 and BC_1F_2-296.

Table 4.8. Kernel quality features of backcross lines evaluated using Near-Infrared Reflectance Spectroscopy (NIRS)

S. No.	Genotypes	Oil content (%)	Oleic acid (%)	Linoleic acid (%)	O/L ratio	Palmitic acid (%)	Protein content (%)
1	BC_1F_2-4	49.7	65.2	11.9	5.4	10.5	25.0
2	BC_1F_2-5	50.7	65.2	10.6	6.1	9.1	24.8
3	BC_1F_2-34	49.2	64.3	8.6	7.4	7.9	25.4
4	BC_1F_2-76	51.2	81.3	6.0	13.4	9.6	27.3
5	BC_1F_2-120	51.3	67.2	10.5	6.3	10.1	26.0
6	BC_1F_2-144	50.7	66.2	12.6	5.2	11.5	26.3
7	BC_1F_2-186	53.2	70.2	8.6	8.1	9.3	27.0
8	BC_1F_2-214	54.5	72.2	9.2	7.7	8.9	25.4
9	BC_1F_2-216	46.9	62.5	11.5	5.4	11.1	28.9
10	BC_1F_2-225	51.8	71.2	10.5	6.7	7.5	26.8
11	BC_1F_2-249	52.1	74.1	8.4	8.7	10.0	25.6
12	BC_1F_2-269	46.0	67.9	11.2	6.0	9.0	23.9
13	BC_1F_2-278	54.9	82.8	5.1	16.1	9.9	29.5
14	BC_1F_2-296	51.2	83.4	4.8	17.1	8.0	26.4
15	BC_1F_2-311	47.7	77.3	9.0	8.5	10.3	23.3
16	BC_1F_2-327	51.9	64.0	11.0	5.7	10.9	28.1
17	ICGV 13193 (DP for FDR)	45.6	44.6	30.1	1.4	13.4	26.8
18	ICGV 15033 (DP for high oleic traits)	52.0	82.4	5.2	15.8	7.03	22.4
19	Kadiri 6 (RP)	49.5	39.8	37.4	1.0	13.3	26.6
	CD (P= 0.05%)	1.6	1.9	1.0	5.5	0.3	1.2
	SEm ±	0.5	0.6	0.3	1.7	0.1	0.4

DP: Donor Parent, RP: Recipient parent, O/L ratio: oleic acid/linoleic acid

The palmitic acid varied from 7.5% to 11.1% with an average of 9.6% indicating a reduction of 2.2-5.8% compared to the recurrent parent (13.3%). This indicates that the selection of high oleic acid progenies will bring about a corresponding increase in O/L ratio and lower levels of linoleic and palmitic acid.

Janila *et al*. (2016a) and Bera *et al*. (2018) observed that the introgression of mutant *ahFAD2* alleles into ICGVs 06110, 06142, 06420 and 05141 leads to increase in oleic acid (≥80.0%) and reduced accumulation of linoleic acid (≤10.0%) and palmitic acid (≤10.0%).

Reddy *et al*. (2017) observed that the frequency distribution of oleic, linoleic, and palmitic acids influenced by a single factor, *i.e*., *ahFAD* alleles. The palmitic acid (C16:0) is a saturated fatty acid contributes about 10.0% of the total oil content while the remaining 10% is contributed by minor fatty acids (Wang *et al*., 2015). Normal groundnut oil with high linoleic acid is vulnerable to oxidation, leading to unpleasant smell, taste and short shelf life of the oil and other groundnut products. In general, human consumption of the groundnut oil with high proportion of oleic acid and low proportion of linoleic acid (high oleic/linoleic acid ratio) is preferable because it cuts down the risk of cardiovascular disease (CVD) by reducing low-density lipoproteins (LDL) levels in the blood (Vassiliou *et al*., 2009).

Hence, the combination of high oil content with high oleic acid, and low linoleic and palmitic acid is desirable for the improvement in oil quality of groundnut. The present study identified three BC_1F_2s-76, 278 and 296 with high oleic acid (≥80.0%) and high oil content (>50.0%) indicating that these segregants can be used to develop a high oleic acid variant of K 6.

4.5.2.3 Protein content (%)

The protein content of the BC_1F_2 progenies varied between 23.3 and 29.5% with a mean of 26.2%. The higher protein content was identified in progenies BC_1F_2-278 (29.5%), BC_1F_2-216 (28.9%) and BC_1F_2-327 (28.1%) in comparison with K 6 (26.6%). In the earlier reports, the protein content in groundnut ranged from 16 to 34% (Dwivedi *et al*., 1990).

4.5.3 Superior segregants for late leaf spot and rust resistance, agronomic, and kernel quality traits

The combined approach of marker-assisted selection coupled with phenotypic-based screening in BC_1F_2s was found suitable and effective in selecting superior segregants for LLS and rust resistance, desired pod features, and improved oil quality traits. The progenies BC_1F_2-76, BC_1F_2-278 and BC_1F_2-296 were found to be at par to marginally superior to the recurrent parent and were selected (Table 4.9). The rust and LLS score of the selected lines at 35 DAI varied from 4.0 to 4.5 and 3.0 to 3.5 respectively, indicating good resistance. The days to 50% flowering in progenies was 35 days in BC_1F_2-76, 34 days in BC_1F_2-278 and 36 days in BC_1F_2-296. The recurrent parent K 6 took 35 days for 50% flowering. The plant height in the progenies ranged from 21.0 to 32.5 cm while it was 34.6 cm for K 6. The number of pods per plant was 38 and 45 for BC_1F_2-76 and BC_1F_2-278, respectively as against 30 for K 6. The selected lines had pod yields ranging from 21.5 to 39.7 g per plant, while it was 26.8 g for K 6.

The oleic acid content in the selected progenies was high and ranged from 81.3 to 83.4 % (Table 4.9). All the selected segregants showed higher oil content (>50.0%), oleic acid (>80.0%) and reduced linoleic acid content (<10.0%) (Figure 4.7 and Figure 4.8). The pod characteristics, pod constriction and pod reticulation were slight in all three selected backcross progenies, pod beak was slight in BC_1F_2s-76 and 296 and medium in BC_1F_2-278, pod ridges were absent in BC_1F_2s-278 and 296 whereas slightly present in BC_1F_2-76 (Figure 4.9).

In earlier reports, MABC has been successful in improving groundnuts for nematode resistance (Simpson *et al*., 2003), rust resistance (Varshney *et al*., 2014), LLS and rust resistance (Sharanabasappa and Bhat, 2015, Janila *et al*., 2016b and Kolekar *et al*., 2017) and enhancing the oleic acid content in groundnut (Janila *et al*., 2016a). Similarly, MABC was deployed to develop pyramided lines in groundnut for nematode and high oleic content (Chu *et al*., 2011); the PRKN and TSWV resistance with the high oleic acid trait (Holbrook *et al*., 2017). However, this is the first study to combine LLS and rust resistance and high oleic traits in groundnut using a marker-assisted selection approach.

Table: 4.9. Late leaf spot and rust reaction, agronomic performance and oil quality features of selected superior segregants

Genotypes	Disease score (35 DAI)		Agronomic traits						Pod characteristics						Oil quality features			
	LLS score	Rust score	DFF	PH	NPP	PY/P	SY/P	SH	PL	PW	PCN	PR	PB	PRG	Oil content (%)	Oleic acid (%)	Linoleic acid (%)	Palmitic acid (%)
BC_1F_2-76	3.0	4.0	35.0	21.0	38.0	28.1	20.5	73.0	18.8	8.7	S	S	S	S	51.2	81.3	6.0	9.64
BC_1F_2-278	3.5	4.0	34.0	29.0	45.0	39.7	29.2	73.6	20.7	9.1	S	S	M	A	54.9	82.8	5.1	9.93
BC_1F_2-296	3.5	4.0	36.0	32.5	28.0	21.5	15.7	73.0	25.1	10.5	S	S	S	A	51.2	83.4	4.8	8.01
ICGV 13193	3.0	3.5	37.0	29.5	41.0	32.7	28.2	74.0	21.4	11.0	S	S	S	S	45.6	44.6	30.1	13.4
ICGV 15033	5.0	4.0	36.0	30.0	33.0	25.3	19.3	76.2	20.5	9.8	S	S	S	A	52.0	82.4	5.2	7.03
Kadiri 6	7.5	7.0	35.0	34.6	30.0	26.8	19.8	73.8	24.5	11.5	M	M	P	M	49.5	39.8	37.4	13.3

DAI: Days After Inoculation, LLS: Late Leaf Spot, DFF: Days to 50% Flowering, PH: Plant Height, NPP: Number of Pods Per Plant, PY/P: Pod Yield per Plant (g), SY/P: Seed Yield per Plant (g), SH%: Shelling Outturn (%), PL: Pod Length (mm); PW: Pod Width (mm), PCN: Pod Constriction, PR: Pod Reticulation, PB: Pod Beak, PRG: Pod Ridges, S: Slight, M: Medium, P: Prominent, A: Absent

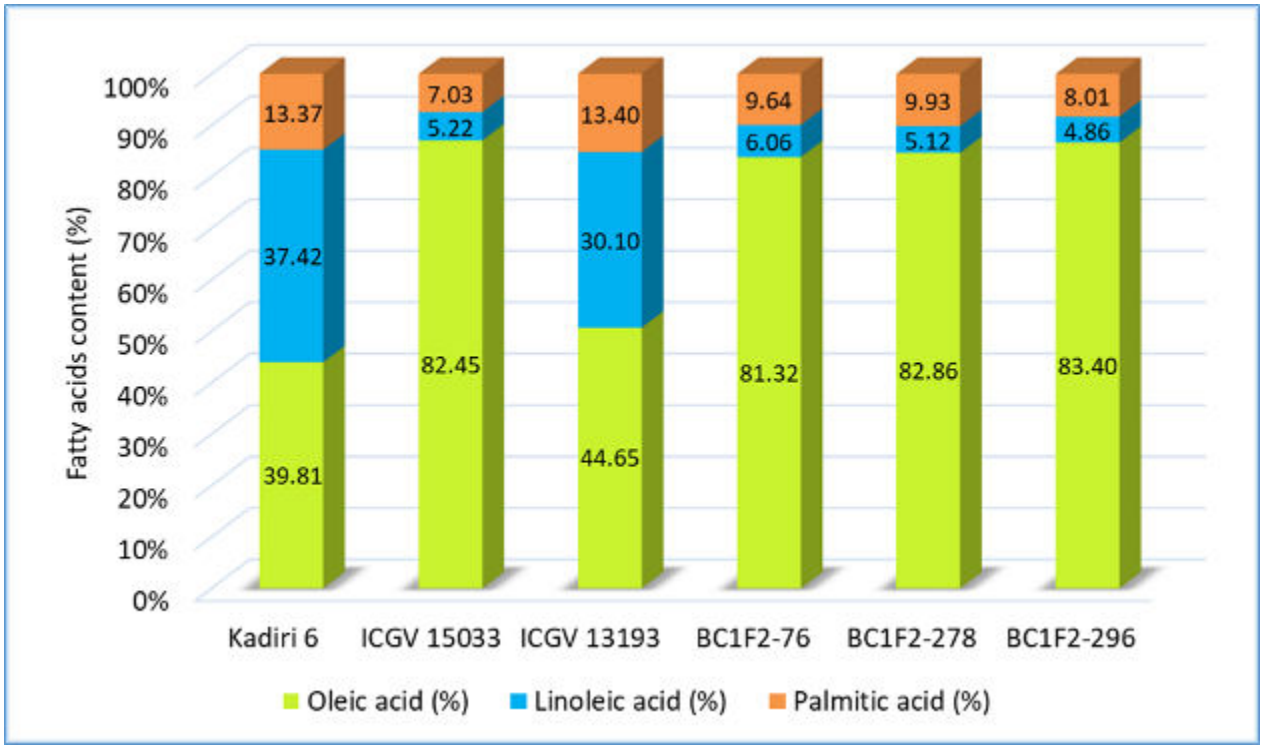

Figure 4.7 Fatty acid profiles of parental lines and backcrossed progenies with increased oleic acid and desirable range of linoleic and palmitic acid.

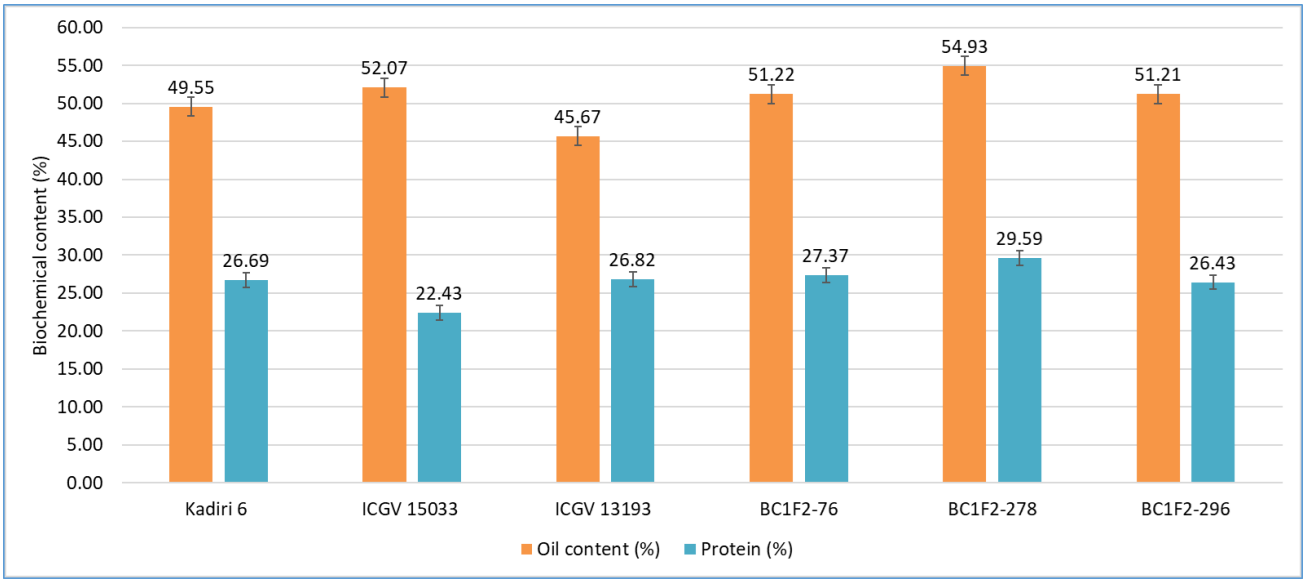

Figure 4.8 Oil and protein contents of selected Kadiri 6 derived backcrossed progenies (BC_1F_2s) with oil quality features *viz*., oil and protein content.

Kadiri 6 (RP) | **ICGV 13193 (DP)** | **ICGV 15033 (DP)** | **BC_1F_2-76** | **BC_1F_2-278** | **BC_1F_2-296**

Figure 4.9 Pod and kernel features of selected Kadiri 6 derived backcrossed progenies (BC_1F_2s).

The major benefit of phenotypic screening coupled with MAS in early generations is that it facilitates selection for a trait/s of interest when little is known about the underlying genes or gene interactions (Hickey *et al*., 2017). Even though, SNP markers confirmed the presence of LLS and rust governing major effect QTLs, little is known about the other minor genes or genetic modifiers that appear necessary to achieve high levels of resistance that is relatively stable across environments.

The LLS resistance is governed by a genetically complex mechanism such as the latent period of the fungus, reduced sporulation and smaller lesion diameters (Dwivedi *et al*., 2002). The rust resistance in groundnut was reported to be due to the accumulation of phytoalexins and upregulation of 1,3-α-glucanases, 1,3-β-glucanases, and proteinases (Sankara *et al*., 1996). A better understanding of the physiological and biochemical responses to these diseases and yield trade-off in resistant cultivars can be future avenues of research. Broadening the genetic base for LLS and resistance can provide the broad spectrum resistance against LLS and rust. This can contribute to improved cultivar selection and modeling growth and yield responses of groundnut to LLS and rust resistance.

The backcross-derived populations developed in the present study displayed improved resistance to LLS and rust and were higher or equivalent yielding in comparison to recurrent parent K 6. Adoption of more efficient breeding strategies that involve the 'fusion' of modern technologies is critical for increasing genetic gain in crop breeding programs (Hickey *et al*., 2017). This approach could be useful for rapid transfer of disease resistance into existing groundnut cultivars that are currently grown by farmers. It should be noted that backcrossed progenies were not evaluated for replicated field trials, thus represents a limitation of this study. Considering the importance of complex traits such as yield and stability, additional cycles of backcrossing may be needed to preserve gene combinations critical for these traits, which are already present in the target cultivar.

Groundnut with the desirable fatty acid profile is key for sustaining the demand of consumers and traders in the coming years. India is the second largest groundnut producer holds the great market potential of high oil and oleic groundnut. When the promising breeding lines from this study become homozygous (F_5 or later), they can be harvested in bulk and evaluated in replicated field trials for disease resistance and yield stability.

Advanced breeding lines derived from K 6 for LLS, rust resistance and high oleic traits will be of significant practical value in providing LLS, rust resistance in K 6 growing

regions, widening the repertoire of kernel quality. The above results also indicate the effectiveness of the molecular marker selection strategy in hastening the process of gene pyramiding/introgression for multiple traits in groundnut.

SUMMARY AND CONCLUSIONS

Chapter V

SUMMARY AND CONCLUSIONS

Kadiri 6 (K 6) is a popular high yielding groundnut variety widely cultivated by farmers in the Indian states of Andhra Pradesh, Telangana and Karnataka and also preferred by traders for its desirable pod and seed characteristics. However, K 6 is highly susceptible to LLS and rust diseases that reduce the pod and fodder yield as well as quality. The work reported here attempts to introgress LLS and rust resistance and high oleic traits into K 6 using marker-assisted backcrossing (MABC) approach. High throughput Kompetitive allele-specific PCR (KASP) SNP genotyping platform helped to genotype the simple and backcross populations in less window period. The developed BC_1F_2 progenies were screened for LLS and rust resistance using detached leaf assay. The BC_1F_2 progenies were evaluated in the field for morpho-agronomic and oil quality traits. Non-destructive near infrared reflectance spectroscopy (NIRS) was used for estimation of kernel quality traits. The major findings of the study are summarised here-

- The donor line ICGV 13193 possessing QTLs for LLS and rust resistance was crossed with K 6. Hybridity test was performed with markers linked to LLS and rust resistance. This cross yielded 74% success rate with 128 true hybrid plants.
- In another cross, K 6 × ICGV 15033 (oleic acid content of ca. = 82%,) a total of 151 true F_1 plants were obtained with 72% success rate.
- Intercross was attempted between two F_1s obtained from two crosses. A total of 520 seeds were harvested from this intercross.
- In KASP genotyping assay, five SNPs for LLS, and rust *viz*., snpAH0015, snpAH0017, snpAH0018, snpAH0021 and snpAH0026 showed a base combination of T:T, A:A, G:G, C:C and G:G for resistant type alleles. Another snpAH002 showed A:A and A:- for homozygous and heterozygous allele call respectively for the *ahFAD2B* allele.
- In the hybridity test of 415 intercross seedlings, KASP assay revealed 44 positive F_1 plants with 11% hybridity output. These plants had heterozygote alleles for rust, LLS and *ahFAD2B*.

- These 44 positive plants were used to make the first backcross with the recurrent parent K 6. From this cross, 420 BC_1F_1 seeds were harvested.
- A total of 380 seedlings were raised. The hybridity test revealed 41 plants with heterozygous target alleles for high oleic acid and foliar fungal disease resistance.
- In summer 2018 season, 343 BC_1F_2 seedlings were raised in the field and screened for zygosity using KASP based SNP markers. The assay revealed 16 plants with homozygous alleles for rust, LLS and high oleic trait.
- The backcrossed progenies of BC_1F_2s were evaluated for LLS and rust response under controlled environmental conditions by using a detached leaf assay method. The score of LLS was recorded with a range of 3.0 to 5.0 in progenies. The progenies *viz*., BC_1F_2s-76, 216, 225 and 311 were found to be resistant for LLS with a score of 3.0. The K 6 and ICGV 13193 were recorded with a score of 7.5 and 3.0 respectively, for LLS.
- The rust disease was scored with a range of 3.0- 4.0 in BC_1F_2 progenies. The recurrent parent (K 6) and ICGV 13193 recorded scores of 7.0 and 3.5 respectively, in the detached leaf assay.
- A set of 343 BC_1F_2 progenies were raised in the field during the summer season, 2018 (March to June) and evaluated for morpho-agronomic features. A number of pods per plant ranged from 24.0 to 58.0 with a mean of 38.5. The progeny, BC_1F_2-269 recorded 58 pods per plant, followed by BC_1F_2-120 with 55.0 and BC_1F_2-144 with 54.0 pods. The recurrent parent K 6 recorded 30.0 pods per plant.
- The mean pod yield per plant was 28.7 g. The highest number of pods was recorded in progeny, BC_1F_2-269 (40.8 g). K 6 was recorded with 26.80 g pod yield.
- The shelling out-turn ranged from 62.3 to 78.9% with a mean of 72.4%. The higher shelling outturn was recorded in BC_1F_2-269 (78.9%) followed by BC_1F_2-186 (78.5%) and BC_1F_2-34 (78.3%).

- In pod features, the mean length of mature pods was measured and it ranged from 18.8 to 27.4 mm with an overall mean of 23.9 mm. The minimum pod length was observed in BC_1F_2-76 (18.8 mm), while maximum pod length was observed jointly in BC_1F_2s-214 and 327 (27.4 mm). Ten progenies (BC_1F_2s- 4, 5, 34, 144, 186, 214, 269, 296, 311 and 327) was measured with pod length similar to K 6. The pod length of K 6 was measured to be 24.5 mm.
- The width of mature pods ranged from 8.7 mm (BC_1F_2-76) to 11.9 mm (BC_1F_2-34). The pod width of K 6 was 11.5 mm. Ten progenies (BC_1F_2s- 4, 5, 34, 144, 186, 216, 225, 269) was measured with pod width on par to K 6.
- A total of 14 progenies recorded slight pod constriction. Two progenies (BC_1F_2-34 and BC_1F_2-327) had moderate constriction similar to recurrent parent K 6. Pod reticulation was slight in 13 progenies and moderate in three progenies. K 6 exhibited moderate pod reticulation.
- Pod beak feature was slight in 14 progenies and moderate in BC_1F_2s-34 and 278. The recipient parent K 6 possess prominent beak.
- Moderate pod ridges were observed in two progenies, slight in seven progenies and absent in seven progenies. K 6 was noted with moderate pod ridges.
- The oil content was ranged from 46.0% to 54.9% with an average of 50.8% in the BC_1F_2 population. The recurrent parent, K 6 had 49.5% oil. The higher oil content (>50.0%) was observed in 11 progenies (BC_1F_2s-5, 76, 120, 144, 186, 214, 225, 249, 278, 296, 327).
- The oleic acid ranged from 62.5% to 83.4% with a mean of 70.9%. The higher oleic acid (>80%) was observed in BC_1F_2-76 (81.32%), BC_1F_2-278 (82.8%) and BC_1F_2-296 (83.4%). A total of five selected progenies (BC_1F_2s-186, 214, 225, 249 and 311) showed above ≥70.0% oleic acid which is considered as moderate to high oleic lines.
- The mean linoleic acid content recorded in the progenies was 9.3% while that of K 6 was 37.4%. The higher O/L ratio (>10) was recorded in three progenies BC_1F_2s-76, 278 and BC_1F_2-296.The oleic: linoleic acid ratio (O/L) was varied from 5.2 to 17.1.

- The protein content of the progenies varied between 23.3 to 29.5% with a mean of 26.2%. The higher protein content was measured in progenies BC_1F_2-278 (29.5%), BC_1F_2-216 (28.9%) and BC_1F_2-327 (28.1%). K 6 was recorded with 26.6% protein.
- Three progenies *viz*., BC_1F_2-76, BC_1F_2-278 and BC_1F_2-296 were selected as superior segregants. These three progenies showed resistance to LLS with a disease score of 3.0- 3.5 and rust resistance with a disease score of 4.0. Pod constriction and pod reticulation were slight in the selected lines. Pod beak was slight in two progenies (BC_1F_2s-76 and 296) and medium in BC_1F_2-278. The pod ridges were absent in BC_1F_2s- 278 and 296 and slight in BC_1F_2-76. All the selected segregants showed higher oil content (>50.0%), oleic acid (>80.0%) and reduced linoleic acid (<10.0%).
- Advanced promising breeding lines identified from the study, following evaluation in replicated field trials for disease resistance, oil quality and yield stability, will be of significant practical value in providing LLS, rust resistance in K 6 growing regions, and also for widening the repertoire of nutritional quality.
- The results of the present investigation demonstrated the effectiveness of a breeding scheme that involves using a molecular marker for early generation selection followed by precise phenotyping for target traits to enhance genetic gain for target traits and improve selection efficiency. Artificial inoculation technique offers a precise screening for rust and LLS in the present study. Early generation selection of target trait-specific markers allowed gene pyramiding for LLS and rust resistance and high oleic acid traits in groundnut thus enabling simultaneous improvement of several target traits in breeding pipelines.

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LITERATURE CITED

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APPENDICES

Appendix A: Weather data of experimental field during post rainy 2018

Month	Rainfall (mm)	Max Temp (°C)	Min Temp (°C)	Mean Temp (°C)	RH max. (%)	RH min. (%)	Mean humidity (%)
March	0.4	35.9	18.3	27.1	64.2	22.5	43.3
April	27.7	37.5	22.0	29.8	70.2	31.2	50.7
May	21.3	39.5	24.7	32.1	66.6	30.9	48.8
June	130.6	34.3	22.9	28.6	81.0	50.8	65.9

RH: Relative humidity