IDENTIFICATION OF MOLECULAR MARKER LINKED WITH BACTERIAL WILT RESISTANCE IN MARIGOLD (*Tagetes erecta* L.)

By

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(2018-11- 003)

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KERALA, INDIA

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THESIS

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CENTRE FOR PLANT BIOTECHNOLOGY AND MOLECULAR BIOLOGY

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KERALA, INDIA

2022

DECLARATION

I, hereby declare that this thesis entitled**"Identification of molecular marker linked with bacterial wilt resistance in marigold (*Tagetes erecta*L.)"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

Vellanikkara

Date: 08.04.2022

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CERTIFICATE

Certified that the thesis entitled **"Identification of molecular marker linked with bacterial wilt resistance in marigold (*Tagetes erecta*L.)"**is a record of research work done independently by **Sreekutty S. S. (2018-11-003)** under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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We, the undersigned members of the advisory committee of **Ms. Sreekutty S. S.** (2018-11-003) a candidate for the degree of **Master of Science in Agriculture** with major field in **Plant Biotechnology**, agree that this thesis entitled **"Identification of molecular marker linked with bacterial wilt resistance in marigold (*Tagetes erecta* L.)"** may be submitted by **Ms. Sreekutty S. S.** in partial fulfilment of the requirement for the degree.

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ABBREVIATIONS

CPBMB	**Centre for Plant Biotechnology and Molecular Biology**
DH	**Double haploid**
DNA	**Deoxyribonucleic acid**
DNase	**Deoxyribonuclease**
dNTPs	**Deoxyribo Nucleoside Triphosphate**
EDTA	**Ethylene Diamine Tetra Acetic acid**
gm	**Gram**
ISSR	**Inter Simple Sequence Repeat**
KAU	**Kerala Agricultural University**
kb	**Kilo base pairs**
MAS	**Marker Assisted Selection**
mg	**Milligram**
ml	**Millilitre**
mM	**Milli Molar**
ng	**Nano gram**
oC	**Degree Celsius**
PCR	**Polymerase Chain Reaction**
pH	**Hydrogen ion concentration**
PVP	**Poly Vinyl Pyrrolidone**
QTL	**Quantitative Trait Loci**
RAPD	**Random Amplified Polymorphic DNA**
RFLP	**Restriction Fragment Length Polymorphism**
RNA	**Ribonucleic acid**
RNase	**Ribonuclease**
TAE	**Tris Acetate EDTA**
TE	**Tris EDTA**

Introduction

1. Introduction

Marigold (*Tagetes* spp., Asteraceae) is an annual flower crop with great commercial potential. Genus *Tagetes* accommodates 55 species (Godoy-Hernandez and Miranda-Ham 2007), of which *T. erecta* L. (African marigold) and *T. patula* L. (French marigold) are commercially important. The diploid (2n =24) *T. erecta* is tall with large flowers, while tetraploid (2n =48) *T. patula* is dwarf with medium, multi-coloured flowers. Marigolds native to Mexico and South America, were first discovered by the Portuguese in Central America in sixteenth century. They introduced these flowers to Europe and India and currently it is widely cultivated in this sub-continent. The major center of marigold production in India is Calcutta region (Mor *et al*., 2008).

Globally marigold is being cultivated in an area of 66.13 thousand hectares with the production of 603.18 thousand metric tons (Mehraj *et al*., 2019). In India, marigold occupies two-third of total loose flower growing and ranks number one in production. It is being cultivated in an area of 66.13 thousand hectares with the production of 603.18 thousand metric tonnes (*Horticulture Statistics at a glance*, 2017). It is very much ingrained into the culture of the people of our country

This flower crop has humpty number of uses as loose flower, as garden plant and also in pharmaceutical, cosmetics, textile industries and also in production of poultry feed. The diuretic, antispasmodic, anti-inflammatory, anti-haemorrhagic, diaphoretic, anthelmintic and carminative properties (Abad *et al*., 1999) of marigold are being utilized in pharmaceutical industries. The rich lutein content adds to its pharmaceutical value (Hojnik *et al*., 2008). *Tagetes* spp. due to the presence of alpha-tertheinyl compound in the roots, are known for repellent activity against root knot nematodes (Somasundaram, 2017).

Apart from the above values, marigold plant also contains numerous allelopathic bioactive compounds that are widely employed as insecticides, fungicides and nematicides (Vasudevan *et al*., 1997; Topp *et al*., 1998; El-Hamawi *et al*., 2004; Romagnoli *et al*., 2005). The species also has the advantage that it can be cultivated under a broad range of climatic conditions.

Bacterial wilt is a very serious disease in marigold and important crops such as Solanaceous vegetables. Bacterial wilt is caused by rod shape, motile, aerobic and soil born Gram negative bacteria, *Ralstonia solanacearum*. This soil-borne disease infects root and stem of the plants, causing a sudden wilting and death. The bacterium has a host range of 200 species and are divided into five races based on its hosts and six bolivars according to their ability to utilize sugar alcohols and disaccharides (Hayward *et al*., 1991; Fegan and Prior, 2005).

In solanaceous plants, yield losses range from 65-70 per cent (Khapte *et al*., 2018) and losses are reported to be increasing drastically year after year (Pandiyaraj *et al*., 2019). This disease often gets worse in Assam, Bihar, Goa, Himachal Pradesh, Karnataka, Kerala, Maharashtra, Orissa and West Bengal, leading to 100 per cent crop losses. In African marigold genotypes, incidence of bacterial wilt has been as high as 62.23 per cent (Mondal *et al.*, 2011). In Kerala, commercial marigold cultivation using bacterial wilt susceptible F_1 hybrids has been leading to heavy crop losses (Jeevan *et al.*, 2020).

The high degree of variation in pathotype and strain among the pathogen has allowed the growing of the varieties tolerant to specific strains, at least in some regions. Since the pathogen is soil borne, its management is also very difficult (Nakaho *et al*., 1996). Hence, breeding of varieties with stable resistance against different pathotypes has become essential. Traditional breeding for bacterial wilt has been proven cumbersome due to the reasons such as variation in pathogen population, low efficiency and impact of environment on disease development. Mitigation of linkage drag has been difficult in conventional back crossbreeding whereas it is possible in molecular marker assisted breeding. Marker Assisted Selection (MAS), selection based on genotype, drastically improves the throughput, with the help of molecular markers that are tightly linked to the trait of interest (Mohan *et al*., 1997). Hence, breeder friendly markers linked to genes/QTL need to be identified for bacterial wilt resistance in marigold.

Over the years, molecular marker technology has evolved to become a useful tool for new varietal generation (Winter and Kahl, 1995). Additionally, it is commercially important for the identification of hybrid purity, genetic diversity and

characterization of varieties in germplasm and for the parental selection in breeding. Amongst the many molecular marker systems available, inter simple sequence repeat (ISSR) marker system carries the advantages good reproducibility, generation of multiple amplicons and greater polymorphism (Powell *et al*., 1996).

The present research program was taken up with the objective to identify an inter simple sequence repeats (ISSR) marker for resistance to bacterial wilt disease of marigold (*Tagetes erecta* L.).

Review of Literature

2. REVIEW OF LITERATURE

The research programme entitled "Identification of molecular marker linked with bacterial wilt resistance in marigold (*Tagetes erecta* L.)" with the objective to develop molecular markers linked to Bacterial Wilt (BW) resistance in marigold. Among the many diseases causing crop failure in marigold, bacterial wilt caused by *Ralstonia solanacearum* is most devastating. Due to the fast spread, it is widely seen in all kinds of environments especially tropical and sub-tropical regions. The bacterium has an extensive host range of over 200 species, which mainly includes solanaceous crops. Thus, there is a necessity for developing bacterial wilt resistant and high yielding cultivars.

1. Marigold as a major crop

Among the loose flowers marigold ranks first in India, commonly grown due to its low cost of cultivation and extensive uses in religious and social functions.

In India, 1,82,900 ha is under floricultural crop cultivation. Marigold covers approximately two thirds of this area, with the annual production of 10,20,600 million tons flowers (ICAR, 2011). According to Mehraj *et al.* (2019), area and production of marigold lies in the first position among the loose flowers in India. From the *Horticultural Statistics at a Glance* (2017), total area of marigold cultivation in 2015-16 was 63.13 thousand ha with 603.18 thousand MT production (Siram *et al*., 2019).

According to APEDA (2016), Madhya Pradesh is the largest producer of marigold (94 tonnes) followed by Karnataka (87 tonnes). Other states which mainly contribute to marigold production are Gujarat (81.7 tonnes), Andhra Pradesh (66.54 tonnes), Haryana (61.83 tonnes), West Bengal (58.1 tonnes), Maharashtra (48.29 tonnes), Chhattisgarh (30.50 tonnes), Tamil Nadu (18.08 tonnes) and Sikkim (18.50).

Even though the marigold production is low in Kerala, due to the unsuitable agro-climatic conditions, it has high demand in Kerala during festival seasons especially during Onam. Threat of diseases especially in humid conditions and lack of awareness about package of practices are the major constraints for marigold production in Kerala (Baburaj *et al*., 2018).

1.1 History of marigold

Marigold is an annual plant, native to America and later migrated to Mexico. In the beginning, marigold flowers were mostly used by Aztec people who were a dominant community in Mexico during the sixteenth century. Aztecs tried to breed the seeds for bigger blooms and later a community named Scuttlebutt took the seeds into France. The production and usage of marigold flowers started initially in Europe during the Bronze Age for pigment production. Later it was brought into Africa, where some got adapted, naturally bred and evolved into a new species called *Tagetes erecta* (African marigold). The flowers were used as therapeutics in Europe for curing intestine and stomach diseases, hence become popularized in medical industry. Later, marigold plants were used as an efficient pesticide.

In India, marigold was introduced from Portugal (Gupta and Vasudeva, 2012) and initially the major area of marigold production was Calcutta, and later brought to New Delhi for trading. Primarily, *T. erecta, T. patula,* and *T. tenuifolia* are grown in India (Mor *et al*., 2008). *T. erecta* of Mexican origin was highly adaptable and easy to cultivate (Kaur and Kaur, 2013; Gurjar *et al*., 2019). During 1980's its production has increased enormously due to the hazardous effects of synthetic dyes in textile industries (Jothi, 2008).

1.2 Botany of marigold

Marigold belonging to the genus *Tagetes* (Family Asteraceae) was first described by Carl Linnaeus in 1753. Almost 50 species are included in this genus and the most commonly grown ones are *T. erecta* L. (African marigold), *T. patula* L. (French marigold) and *T. tenuifolia* Cav. (Striped marigold) (Panwar *et al*., 2017). The main mode of propagation in marigold is sexual reproduction while asexual reproduction is also possible using stem cuttings (Mehraj *et al*., 2019).

African marigold varies in colour from yellow to orange and grows tall up to three feet. French marigold usually grows 5-18 inches tall and seen in red, orange and yellow shades (Priyanka *et al*., 2013).

African marigold grows straight upward to a certain height and later develop lateral branches with flowers. Plants take 45 days to flower and as a short-day plant, main flowering happens during winter and rainy seasons. Rainy and winter season crops are transplanted in July-August and September-October, respectively (Gupta and Vasudeva, 2012; Mailem and Singh, 2018).

African marigold has large, yellow or orange flower heads whereas French marigold has smaller, yellow or orange and red, single or double heads (Pramila *et al*., 2011). Male sterile lines were also used in the hybridization programmes even though the development of MS lines is highly time-consuming (Wang *et al*., 2010). Molecular markers make the breeding programmes more reliable (Namita *et al*., 2013).

1.3 Diseases of marigold

Though having an insect repellent property, marigold is affected by a number of bacterial and fungal diseases. Some of the important diseases of marigold are flower blight (*Alternaria zinniae*), bacterial wilt (*Ralstonia solanacearum*), wilt and stem rot (*Phytophthora cryptogea*), collar rot (*Phytophthora* sp.; *Pythium* sp.), damping off (*Pythium* sp.), *Alternaria* leaf spot, *Fusarium* wilt (*F. oxysporum*), *Cercospora* leaf spot (*C. megalopotamica*) and bacterial leaf spot (*Pseudomonas syringae*). Aster yellows is one of the viral diseases affecting the marigold. Phytoplasma and nematode diseases are another group of diseases that affect marigold (Gurjar *et al*., 2019).

2. Bacterial wilt

Bacterial wilt is a major disease causing yield loss, especially in case of Solanaceous crops. In Kerala, bacterial wilt is a serious threat to marigold cultivation, and hence it is necessary to breed new varieties with resistance.

The early reports of bacterial wilt in crops were from the regions of Asia and South America, during the 19th century. Crops mainly affected were potato, tobacco, tomato and groundnut. Initially the causative bacterium was described as *Pseudomonas solanacearum* and later it was identified as *Ralstonia solanacearum* in

Coleus barbatus in Brazil (Smith, 1896; Yabuuchi *et al*., 1995; Netto and Assis, 2002).

The symptoms appear as a sudden wilt in foliage followed by wilting of the entire branch. Wilting starts appearing from the lower leaves, spreads all over and cause stunted growth. At later stages, recovery is impossible and plants die within 3-4 days. Brown discolouration occurs on the stem as well as branches because of the blockage of water from roots to leaves (Walker, 1952). It takes almost 2-8 days for the symptoms to appear in a plant, as the bacteria first enters into the intercellular spaces of cortex and later into xylem vessels (Chupp and Shurf, 1960).

Bacterial wilt caused by *Ralstonia solanacearum* in banana cause total damage and yield losses. The symptoms appeared on the second or third leaf with crumpling of the lamina and petiole, accompanied by yellowing and death of the whole plant (Gnanamanickam *et al*., 1979). This pathogen is highly destructive in solanaceous plants especially in tomato (Sarkar and Chaudhuri, 2016). Yellowing and wilting are the main symptoms in ginger (Cosmas *et al.* 2016).

In India, West Bengal recorded high incidence of bacterial wilt in marigold (2.76 – 62.23 per cent), during July to October (Shekhawat, 1992; Mondal *et al*., 2011; 2014; Khapte *et al*., 2018). Bacterial wilt is the most disastrous disease of marigold in Andaman Islands with 100 per cent disease incidence in tomato and marigold compared to 55.6 per cent in brinjal and 22.3 per cent in chilli (Sakthivel *et al*., 2017). Nimisha (2016) reported a high incidence of bacterial wilt under Kerala conditions.

2.1 Pathogen for bacterial wilt

Ralstonia solanacearum is the causal organism of bacterial wilt in marigold. These are rod shape, motile, aerobic and soil born Gram negative bacteria (Bhandari, 2018). The bacterium survives in soil at a dormant state for up to six years without depending on any host (Chester, 1950). *R. solanacearum* may spread from one plant to another by several means, such as infected planting material, soil water flow, contaminated soil, field equipment *etc.* (Louws *et al*., 2010). Usually, the pathogen enters the plant through wounds or openings present in the root cells, enters into the

plant cell via cortex and invades the xylem tissues, causes blockage in the vascular system leading to death of the plant (Lebeau *et al*., 2010; Meng, 2013).

The diagnosis for bacterial wilt is done by ooze test in which the vascular discolouration is checked in the stem regions and ooze out confirms the infection (Bangi *et al*., 2020). The bacterial colonies can be isolated by using triphenyl tetrazolium chloride (TZC) which distinguish white colonies with pink bacterial centres (Husain and Kelman, 1958).

The bacterium has a host range of 200 species and divided into five races based on its hosts and six bolivars according to their ability to utilize sugar alcohols and disaccharides (Hayward, 1991; Fegan and Prior., 2005). A new hierarchical classification which divides *Ralstonia solanacearum* into four phylotypes based on both molecular and phenotypical characteristics has led to a better understanding about the pathogenic races (Fegan and Prior, 2005; Murugaiyan *et al*., 2011).

Ralstonia pseudosolanacearum is another bacterium that causes wilt in many plants and blast disease (mahali) in ginger. The species is highly aggressive in nature and has been reported in a wide range of hosts. Another species *R. solanacearum sensu lato* is the most serious threat to the members of Solanaceae, Musaceae and Zingiberaceae families (Prameela and Bhai, 2020).

2.2 Severity and losses

Even though the pathogen possesses nearly two hundred plant species as hosts, suitable management strategies are not available. Due to its inhibitory activity of beneficial bacteria in the rhizosphere region, antibiotics or bacteriocins cannot be used for control of the pathogen (Alamer *et al*., 2020). Bacterial wilt cannot be controlled by the usage of chemicals because pathogen may reside in the soil for many years and attack when favorable conditions arise. Soil fumigation and crop rotation are also not that effective especially in humid tropics. Hence, the only solution is to create resistant cultivars (Nishi *et al*., 2003). Marker-assisted breeding offers assured resistance in the improved lines even though it is time consuming to identify molecular markers associated with the resistance (Kumar *et al*., 2018).

In Karnataka, significant incidence was found in Chikkaballapur (27.60 per cent) followed by Dharwad (22.51per cent) whereas Mysuru had least infection level (4.62 per cent) (Bangi *et al*., 2020).

In Andaman Islands, cross infectivity of bacterial wilt pathogen (*Ralstonia solanacearum*) from marigold was tested in tomato, brinjal and chilli. Tomato and marigold showed 100 per cent incidence compared to 55.6 per cent incidence in brinjal and 22.3 per cent by chilli (Sakthivel *et al*., 2017).

In solanaceous plants, yield losses range from 65-70 per cent (Khapte *et al*., 2018) and losses increase drastically every year (Pandiyaraj *et al*., 2019). This disease often gets more severe in areas like Assam, Bihar, Goa, Himachal Pradesh, Karnataka, Kerala, Maharashtra, Orissa and West Bengal, the damage may also reach up to 100 per cent. Endemically the pathogen is seen in the sub humid areas of Himachal Pradesh every year (Sood and kumar, 2015). Pseudomonas species mainly cause damage in the tropical and subtropical countries and many a times act as a limiting factor for crops like tomato, potato and eggplant (Grimault and Prior, 1993).

Narayanankutty *et al* (2015) studied bacterial wilt incidence in tomato and found that the variety Pusa Ruby exhibited 100 per cent disease incidence. While Shakthi, which was used as a resistant check, showed less than 20 per cent bacterial incidence. The accession LE25 had no incidence and 14 lines showed moderate incidence.

Among two varieties, one local accession and five F_1 hybrids evaluated at KAU, the local accession M_1 was found to be resistant and 100 per cent wilting was shown by the F_1 hybrid Sakura 031. But M_1 plants were having low quality flowers and poor yield (0.234 kg/plant) (Umesh *et al*., 2018).

3. Conventional management of bacterial wilt

The management for bacterial wilt includes cultural, chemical, biological and integrated methods. But these methods were not so effective due to its highly disseminating nature. In ginger, the management strategies were the selection of disease-free rhizomes or microrhizomes, selection of infection-free fields, raised beds

with deep drainage, pre-treatment of rhizomes with plant protection chemicals and strict phytosanitary measures. Biological methods include weed control and crop rotation with non-host plants such as sweet potato and taro (Prameela and Bhai., 2020).

In case of banana, bacterial wilt (*Xhantomonas campestris pv. musacerum*) usually causes yield loss up to 100 per cent in areas of East and Central Africa. Cutting down of affected pseudo stem, cutting down and digging up whole mate of affected plant, bury the plant remains, removal of the bud of affected plant with fork stick are the main cultural practices adopted to manage the disease. Cultivars Abate, Arkya, Heila, Mezya and Sorpie, which are resistant to bacterial wilt were also developed (Geberewold, 2019).

Alcoholic leaf extract of *Lantana camara*, incubation of fresh organic matter of *Cajanus cajan* and *Crotalaria juncea*, rhizome extract of *Cucurma longa*, aqueous extracts of *Adathoda vasica* and *Tagetes patula* and essential oil extracted from *Thymol* spp. and *Cymbopogon martini* can be used for managing the growth of *Ralstonia* in Solanaceous plants especially in tomato (Bhandari, 2018). The phytoextracts of marigold species *T. patula* and other plants *C. procera* and *A. vasica* were effective to control up to 60 per cent of bacterial population in tomato. High dosages (40 g/ kg of soil) of the biocides decrease the gravity of the disease and increases the yield (Din *et al*., 2016).

The *in vitro* assays in potato had shown that the most suited conditions for *Ralstonia solanacearum* are 1×10^2 CFU ml^{-1} concentration, 6-8 leaf stage and incubation temperature of 28ºC. Low SDI (Stem Disease Index) under these conditions was seen in cultivars Nagasaki Kogane, Saikai 35, Meiho, and Norin, indicating higher resistance. Cultivars Aiyutaka, Dejima, and Kennebec showed higher SDI and susceptibility (Habe, 2018).

Essential oils from neem, spearmint, castor, marigold, calamus, olive, turpentine, eucalyptus and garlic, at 5, 10 and 20 mg ml^{-1} concentrations with streptocycline and copper oxychloride mixed had shown that under *in vitro* conditions, neem (20 mg/ml) is best to manage the bacterium (up to 7.20 mm). At 5 mg/ml, garlic showed maximum control up to 5.17 mm (Sood and kumar, 2015).

Integrated management strategy including priming of rhizomes, soil treatment and use of biocontrol agents, is effective to manage the disease in ginger (Prameela and Bhai, 2020). Real time LAMP (Loop-mediated isothermal amplification) can assure disease free planting material. The use of methyl bromide (3 lb/100 ft^2) along with soil fumigation can control bacterial wilt.

Studies with vinegar residue substrate (VRS) and peat substrate showed that VRS not only controlled the disease but also decreased the fungal population, enhanced tomato seedling growth, increased number of beneficial bacteria and actinomycetes and modulated the defense mechanism. By using both of the alternatives, a systematic acquirement of disease resistance was shown by the plants through modulation in transcription factors (He *et al*., 2020).

Avirulent strains of *Bacillus* spp., *R. solanacearum*, *Streptomyces* spp., *Pseudomonas* spp., *Paenibacillus* sp., *Burkholderia* sp. and *Acinetobacter* sp. can be used as biocontrol agents against this bacterium. Organic matter amendments such as animal waste, plant residue and simple organic compounds reduce the bacterial wilt in plants (Yuliar *et al*., 2015).

Marigold itself is an excellent conventional alternative for managing the bacterial wilt in tobacco. The disease index decreased drastically upon intercropping with marigold to 30.12 per cent from 58.25 per cent in tobacco monocropping. The population of beneficial bacteria such as *Burkholderia*, *Mortierella*, *Trichoderma* and *Lysobacter* also got enhanced under intercropping (Li *et al*., 2020). Marigold contains broad spectrum biocides such as 5-(3-buten-l-ynyl)-2,2'-bithienyl (BBT) and 5-(4-acetoxy-l-butynyl)-2,2'-bithienyl (BBTOAc), which has an inhibitory effect on *Ralstonia* (Terblanche and de Villiers, 1998).

3. 1 Managing the bacterial wilt in marigold

Nimisha (2016) reported the incidence of *Ralstonia solanacearum* in African marigold under Kerala conditions. Grafting the four-week old scion from susceptible cv. Maria-91 on resistant six-week old rootstock of cv. M_1 was most promising (Baburaj *et al*., 2018).

Evaluation of eight African marigold accessions (5 F1 hybrids, 2 varieties and 1 local collection) for bacterial wilt resistance under sick plot conditions had shown that P-4 and Sakura 031 hybrids with high yield potential are highly susceptible but local collection M_1 is resistant (Umesh *et al*., 2018).

Evaluation of eight F_1 hybrids, eight *T. patula* genotypes (3 varieties and 5 local collections), seven *T. erecta* local collections and one *T. tenuifolia* genotype, by artificial screening, had shown that two local *T. erecta* lines, M_1 and M_2 are resistant. Compared to monsoon, disease severity was more in winter. It was observed that the flavonoid content of leaves and petals were inversely proportional to the bacterial wilt resistance (Jeevan *et al*., 2020).

4. Breeding for bacterial wilt resistance

Barnes and his co-workers (1971) have identified the alfalfa lines A-C4 and B-C4 and subjected to recurrent selection by phenotypic means. Inter-crossing among 150 plants in each population has reduced the wilting index of A-C4 population from 3.72 to 2.53, 1.38 in subsequent generations. In B-C4, it reduced from 4.25 to 3.94, 3.67, 3.13, and 2.63.

In Kerala, Ramachandran *et al.* (1980) have identified 36 bacterial wilt resistant tomato lines.

Since the host resistance mechanism keeps changing due to the mutation happening in the pathogen, Lebeau *et al.* (2011) suggested a pathoprofile concept having an accession which overcomes most of the pathogen strains. Subsequent studies had shown that significant amount of interaction exists between the crop and environment, creating a variable pathogen population.

Bulked Segregant Analysis (BSA) in an F_2 mapping population of tomato derived by crossing the resistant cv. Anagha with susceptible cvs. DVRT-1 and Pusa Ruby using OPAH, OPS, OPAZ, OPY and OPF primer series, has yielded marker by OPS16 primer in DVRT-1 susceptible parent. Resistant gene in Anagha is proposed to be recessive (Ragina and Sadhankumar, 2013).

Kerala Agriculture University has developed four bacterial wilt resistant varieties Sakthi, Mukthi, Anagha and Manulakshmi. Attempt to pyramid the bacterial wilt and Tomato Leaf Curl Virus (ToLCV) diseases in tomato using bacterial wilt resistant cv. Sakthi and ToLCV resistant line IIHR 2196 had shown that the markers TSCARAAG/CAT was linked with bacterial wilt resistance and Ualty 16 with ToLCV disease (Belge *et al*., 2014).

Kim *et al* (2018) conducted a whole genome resequencing study to find out the SNPs in the genes and identified four genes with polymorphism, responsible for bacterial wilt resistance. SNP markers were developed from these four genes. One among them with a functional SNP in the gene, *Solyc12g009690.1*, was highly effective against bacterial wilt.

Eggplant genotypes IIHR-7, IIHR-500A and CARI-1 were identified to have resistance at field level. Phylogenetic analysis using 37 markers has differentiated the resistant and susceptible genotypes (Khapte *et al*., 2018). Similarly, eggplant accessions BB 64, BB 54, BNDT and PPC were found to have resistance whereas Mgt, Mlp and BR 14 were susceptible and cv. Kasral had medium resistance. In case of F_1, the crosses Mgt x BB 54 and Mlp x BNDT showed resistance and BR 14 x BNDT, BR 14 x PPC and BR 14 x Kasral were susceptible (Kurhade *et al*, 2020). Bacterium was artificially inoculated in eight eggplant cvs. Nayantara, Singhnath, Dhundul, Kazla, Marich Begun, Luffa-s, Kata Begun and Uttara. Highest incidence rate was shown by Luffa-s (80 per cent) while lowest was in Kata Begun (30 per cent) (Rahman *et al*., 2011). Artificial inoculation with bacterial wilt suspension in 50 eggplant varieties through soil drenching and leaf axil puncturing had shown that Arka Keshav, Surya, Arka Neelkanth and Arka Nidhi are resistant (Sadarunnisa *et al*.,2018).

4.2 Breeding marigold for bacterial wilt resistance

Evaluation of eight African marigold accessions (5 F_1 hybrids, 2 varieties and 1 local collection) for bacterial wilt resistance under sick plot conditions had shown that P-4 and Sakura 031 hybrids with high yield potential are highly susceptible but local collection M_1 is resistant (Umesh *et al*., 2018).

Evaluation of eight F_1 hybrids, eight *T. patula* genotypes (3 varieties and 5 local collections), seven *T. erecta* local collections and one *T. tenuifolia* genotype, by artificial screening, had shown that two local *T. erecta* lines, M_1 and M_2 are resistant. Compared to monsoon, disease severity was more in winter. It was observed that the flavonoid content of leaves and petals were inversely proportional to the bacterial wilt resistance (Jeevan *et al*., 2020).

5. Methods of artificial inoculation

Artificial inoculation methods may depend on many characters such as concentration of the inoculum, age of the plants, congenial environment, place where the plants are kept and the reaction of the host plant. Winstead and Kelman (1952) used stem puncture method to artificially inoculate the tomato seedlings with bacterial wilt and to develop the symptoms in 14 days.

Inoculation methods such as seed-soaking in inoculum, seed-sowing followed by inoculum drenching, petiole-excision inoculation, soaking of planting medium with inoculum either directly or after imparting seedling root-injury were compared in tomato using pure bacterial inoculum having 0.1 OD at 10^8 cfu ml^{-1}. The results showed that petiole inoculation is the best method in two-weeks old seedling (Thomas *et al*., 2015).

Artificial inoculation methods media drenching, root dip and stem injection studied in African marigold for bacterial wilt inoculation had resulted same level of incidence (Umesh *et al*., 2018).

By making wounds in the leaf axil of *Strelitzia* seedlings and pouring 30 µL of bacterial suspension over the wounded tissue, twenty-four strains of *Ralstonia solanacearum* races 1, 2 and 3 of bolivars I, II and III were inoculated. Wilt symptoms were shown by the strains from Musa or Heliconia plants (Rodrigues *et al*., 2011).

Among the bacterial wilt artificial inoculation methods, axil puncturing, leaf clipping and soil drenching tested in chilli, brinjal and tomato, soil drenching was

found to give highest incidence (98.0, 90.0 and 95.0 per cent in tomato, chilli and brinjal, respectively) (Artal *et al.,* 2013).

6. Molecular markers for disease resistance

In crop plants, selection based on the morphology was practiced for several years. But environmental influence may badly affect the results while using morphological parameters as selection criteria. Molecular markers provide a quick and reliable method to assess and compare the genotype (Panwar *et al*., 2017; Duarte *et al*., 2018).

Identification of markers linked with bacterial wilt will enable development of resistant varieties in marigold through marker assisted selection (MAS) and also give idea on genes and pathways involved in the resistance mechanism.

6.1 Molecular markers in marigold

Mehraj *et al.* (2019) have screened 36 microsatellite markers for checking the clonal fidelity of marigold developed by double haploidization. RAPD and AFLP markers were used to assess the divergence in marigold. AFLP markers were found to yield 85.71-100 per cent polymorphic bands (Pramila *et al*., 2011; Panwar *et al*., 2017). Mor *et al*. (2008) also have shown that the RAPD markers are capable to assess the genetic diversity among marigold accessions. RAPD markers were also used for identification of male sterility. While using 64 markers, marker G-02^{980} (5'-GGCACTGAGG-3') was found to segregating with the fertility (Wang *et al*., 2010).

Twenty African marigold genotypes, including six Thai landraces and 14 commercial varieties, were evaluated using the microsatellite primers identified through SSR-enriched genomic DNA library, inter-SSR technique and SSR primers transferred from sunflower. These markers had shown that the Thai landraces are closer to African genotypes, than the French genotypes (Whankaew *et al*., 2014).

6.2 Molecular markers for bacterial wilt

Genome-wide SNP analysis was conducted in two groups of tomatoes, one with seven bacterial wilt resistant genotype and other group with two susceptible varieties,

to find out the marker associated with resistance. Resistance QTLs *Bwr6* and *Bwr12* were used to select the SNPs associated with resistance. Analysis on 5259 non-synonymous SNPs has identified 265 SNPs in the coding regions. Leucine-rich repeat (LRR) receptor-like proteins were encoded by four genes in the chromosome 12, which were validated and developed as SNP markers. The SNP marker containing an SNP in the gene *Solyc12g009690.1* was strongly linked with the resistance (Kim *et al*., 2018).

In a gene pyramiding study, of the SCAR markers tested, TSCARAAG/CAT was specific for bacterial wilt and Ualty16 for ToLCV (Belge *et al*., 2014). Using the highly resistant accessions RIL-118, Indam-1004, Arka Samrat, PKM-1, PED, EC-802390, EC-816105, high yielding and resistant variety Arka Rakshak and field susceptible lines, Kumar *et al.* (2018) have identified a marker for bacterial wilt resistance in tomato. The SCAR marker, SCU176- 534 was also found to be associated with bacterial wilt resistance in tomato.

Two segregant populations of eggplant, developed by crossing CARI–1 and IIHR–7 as resistant parents and Rampur Local and Arka Kushmakar (IIHR–108) as susceptible parents, were subjected to BSA using 390 SSR markers. Markers generated with emb01D10, emh11I06, emh02E08, and SSR–46 were identified and used in further breeding programmes (Pandiyaraj *et al*., 2019).

Molecular linkage map for resistance to the bacterial wilt in carnation, caused by *Burkholderia caryophilli*, was constructed using 134 F_1 lines generated from the cross of Carnation Nou No.1 resistant line and Pretty Favvare, susceptible line. STS-WG44 marker linked with the QTL was identified and used in MAS to develop a resistant variety Karen Rouge (Yagi, 2013).

Using marigold resistant line W6 and susceptible lines Hatano and Michinoku, AFLP markers linked to bacterial wilt resistance was identified. The QTL comprising 15 markers was found to contribute to 30 per cent of the variance in resistance among the check lines (Nishi *et al*., 2003).

6.3 ISSR marker system

Inter Simple Sequence Repeats (ISSR) is a universal markers system generating the markers of 100-3000 bp size and lies between two microsatellites. In ISSR marker system, microsatellite sequences are usually taken as primers with some non-repeat adjacent regions as anchors. About 10-16 fragments will be generated from multiple loci simultaneously. Universal primers, requirement of only low quantity of DNA, capability for genome wide analysis, high reproducibility and high sensitivity make this system preferable (Bornet *et al*., 2004). It overcomes some limitations of other random markers such as low reproducibility of RAPD, high cost of AFLP and the need to know the flanking sequences to develop species specific primers as in case of microsatellite markers (Ratnaparkhe *et al.,* 1998; Reddy *et al*, 2002; Ajal *et al*., 2014; Duarte *et al*., 2018).

Using ISSR markers, 160 germplasm of *Dioscorea*, collected from different regions were assessed for genetic diversity (Nudin *et al*., 2017). Mazid *et al.* (2013) conducted a study to find out heritability, variation, divergence and resistance against bacterial blight in different rice genotypes using ISSR markers. About 310 polymorphic loci were identified by using 20 ISSR markers. Quantitative traits which are associated with molecular markers were identified using Mantel test and analyzed the correlated markers for the future breeding programme.

Vijayan *et al*. (2006) have conducted yield trait analysis among 44 mulberry genotypes. ISSR marker analysis have clustered the genotypes into five groups, and step-wise multiple regression analysis has identified the markers associated with each of the yield parameters. Similarly, ISSR markers were useful to find out the genetic diversity among different species of *Trifolium* genus (Rizza *et al*., 2007; Dabkeviciene *et al*., 2011) and chrysanthemum (Indu *et al.,* 2020). ISSR and RAPD analyses were also successful to assess the stability of the micro propagated plants of banana cv. Nanjangud Rasabale (Venkatachalam *et al*., 2007). In *Bombyx mori,* ISSR markers were more powerful to reveal the polymorphism (76.98 per cent) compared to the RAPD and RFLP markers (Nagaraju *et al*., 2001).

6.4 ISSR markers in marigold

Namita *et al.* (2013) screened the genetic diversity of *Tagetes sp* using ISSR and RAPD markers. ISSR markers showed high polymorphism (60.48 per cent) compared to RAPD markers (57.72 per cent) even after using more RAPD markers. URP and ISSR markers were used for the DNA fingerprinting in African marigold (Panwar *et al*., 2018).

Marker associated with a male sterile recessive gene, *tems* was identified using 38 ISSR primers and 170 SRAP primer combinations, from a marigold F_2 population developed by crossing the male sterile line M525A and the inbred line f53f. This marker has been successfully converted into a SCAR marker located at 2.4 cM from the *tems* locus (He *et al*., 2009).

Single marker regression analysis has revealed two ISSR markers, ISSR 811 and ISSR 817, for identification of mutant marigold genotypes. Putative mutant-3 showed dissimilarity from the parent while pm5 and pm6 were having similar genotypes (Majumder *et al*., 2018).

7. Bulk Segregant Analysis (BSA)

In BSA, plants from segregating populations are grouped according to the phenotypical expressions and extreme phenotypes of the targeted trait shall be selected and used for checking the allele differences among the population bulk. Hence, we can access the marker associated with the trait without forming a map (Quarrie *et al*., 1999; Magwene *et al*., 2011).

Quarrie *et al* (1999) stated that BSA is mainly used to detect the allelic status of quantitative traits by using the phenotypical expression of the trait. To improve drought resistance in maize, they phenotypically selected the resistant and susceptible plants and DNA was bulked to find out the polymorphic alleles. Michelmore (1991) used RAPD markers to tag the downy mildew resistant gene in lettuce. Similarly, RAPD marker system was used to dissect the complexity of *Brassicca napus* species through BSA (Dulson and Ripley, 1998).

Salunkhe *et al* (2011) described BSA as an effective strategy to locate the QTLs for drought resistance in rice. RM8085 was located in the QTL region of chromosome 1 at 3.8 cM. Using BSA on 490 RILs created from a cross between Apo and Swarna rice varieties, QTLs associated with lowland drought stress were identified. Two microsatellite markers RM324 and RM416 were found to be associated with drought resistance (Venuprasad *et al*., 2009). When Whole population genotyping, selective genotyping and BSA were compared for their efficiency to identify the markers for drought tolerance in rice, BSA was found to yield higher data points and allele identification. Thus, BSA can be used as a rapid method for identification of QTLs (Vikram *et al*., 2012).

A cross between Q21861 and Galleon were used to create an F_2 mapping population for the identification of RAPD markers linked with resistance to barley leaf rust (*Puccinia hordei*). Marker identified by the primer OPU02, located near the resistance gene *Q21861* was found segregating with the resistance (Poulsen *et al*., 1995).

Two methods such as microarrays and extreme array mapping (XAM) techniques were also used in BSA. Single feature polymorphisms (SFP) were used to detect SNPs. Sequence information availability in *Arabidopsis thaliana* made SFPs unnecessary and instead the arrays ATTILE1R and ATSNPTILE were good for BSA in F_2 population (Becker *et al*., 2011).

Liu *et al* (2020) demonstrated BSA in wheat for stripe resistance, using 141 kompetitive allele-specific PCR (KASP) and 165 SSR markers on chromosome 2B. They successfully located *YrZl31* responsible for stripe resistance on chromosome 2BL.

7.1 Screening for bacterial wilt

Bacterial wilt resistant carnation line Carnation Nou No. 1 and susceptible line Pretty Favvare were crossed to develop an STS marker linked with bacterial wilt resistance. Using BSA, eight RAPD markers were identified and reported to be closely associated with one of the resistant genes. RAPD marker was successfully converted into STS marker and was used in MAS (Onozaki *et al*., 2004).

Mapping the bacterial wilt resistance in carnation using SSR markers was done using BSA in 90 F_2 plants resulted from the cross of resistant line 85-11 and susceptible Pretty Favvare. The map covered 843.6 cM and average distance among two loci was found to be 6.5 cM. Upon QTL analysis, B4 locus was found to be linked with bacterial wilt resistance and represented 63 per cent of the phenotypic variance to resistance. The resistant locus 85-11 was similar to that in the highly resistant wild species, *Dianthus capitatus* (Yagi *et al*., 2012).

BSA has identified a SCAR marker for bacterial wilt resistance in tomato cv. Hawaii 7996. Screening of 800 RAPD primers was done against R pool which represented six resistant F_9 RILs and S pool which represented six susceptible F_9 RILs. Six primers have shown polymorphism of which two primers gave 100 per cent linkage of trait in each plant. SCAR marker SCU176-534 was created from UBC176 and it was further used in selection of resistant lines in breeding programme (Truong *et al*., 2015).

QTL mapping for bacterial wilt resistance was done in peanut through BSA in F_2 mapping population. SNPs associated with bacterial wilt were identified by using 30 each resistant and susceptible F_2 plants, and their respective parental population. Two QTLs *qBW*-1 and *qBW*-2 were identified to be associated with resistance genes and were later confirmed in F_8 RIL population. Four SNPs and one SSR marker were identified in *qBW*-1 (Zhao *et al*., 2016).

BSA was done in mung bean to find the QTLs associated with powdery mildew resistance using $F_{2:7}$ and $F_{2:8}$ RILs derived from resistant V4718 and susceptible CN72 lines and 40 ISSR-RGA marker combinations. Corresponding 10 ISSR markers, 873 ISSR and 756 ISS-RRGA loci has been amplified. Of these, 11 ISSR-RGA loci were found associated with resistance and from that three were associated with powdery mildew resistance gene, along with five ISSR markers. So, a total of eight markers were used in further analysis and identified a major QTL, *qPMC72V18*-1 situated in the flanking position of I42PL229 and I85420 markers (Poolsawat *et al*., 2017).

BSA in 226 F_2 marigold population, generated by crossing male sterile line IIHR10521AB and male fertile line IIHRMY7, has identified a marker linked with

male sterility gene. The SCAR 4 marker was proven linked with sterility locus and was confirmed by using 12 apetalloid male sterile lines (Asha *et al*., 2019).

Materials and Methods

3. MATERIALS AND METHODS

The research entitled "Identification of molecular marker linked with bacterial wilt resistance in marigold (*Tagetes erecta* L.)" was carried at Centre for Plant Biotechnology and Molecular Biology, College of Agriculture during the year 2019 to 2021. The materials used and methods followed for this study are discussed in this chapter.

3.1 Development of mapping population

An F_2 population segregating for the bacterial wilt resistance was developed and used for the ISSR based Bulked Segregant Analysis (BSA) for identifying the molecular markers linked with resistance

3.1.1 Parental population

Two genotypes distinctly differing in their response for bacterial wilt and belonging to *T. erecta* were used as parents in this study. *Tagetus erecta* accessions, KAU- M_1, proven resistant against bacterial wilt (Umesh *et al*., 2018 and Jeevan *et al,* 2020), was taken as resistant parents. Double Yellow, highly susceptible to bacterial wilt, was taken as susceptible parent (Plate 1 a and b). The seeds were collected from the Department of Floriculture and Landscape Architecture, College of Agriculture, Thrissur.

The experimental plot was laid out at the Department of Floriculture and Landscape Architecture (22.25 m above MSL, 10º 31'N latitude and 76º 13' E longitude).

The seeds of the parental lines were sown on July 2019. Seeds were sown in portrays containing cocopeat, vermiculite and perlite in the ratio of 3:1:1. One-month old seedlings were transplanted into pots containing pre-sterilized soil mixed with cow dung in the ratio 3:1 and watered daily. Pinching was done at two-weeks after transplanting.

Fertilizer application was done in accordance with recommended dosages (112.5:60:30 kg/ha N, P_2O_5 and K_2O) in KAU Package of Practice (2016) for

marigold. Half a dose of N, full dose of P_2O_5 and half K_2O were given as a basal dose and the remaining were given in two equal top dresses.

3.1.2 Cross pollination

Susceptible variety Double Yellow was crossed with pollen from the resistant lines KAU -M_1. Flower buds of both female and male parents were bagged in butter paper covers when these buds started showing color by the initiation of opening. Petals surrounding the floral organs were slightly trimmed so that the stigma and anthers were exposed to facilitate easy pollination. The outer ray florets of the female parent were pollinated with the pollen collected from the male parents using a pencil brush. The pollinated flowers were then covered with butter paper bag safely to avoid pollen contamination (Plate 1c). The procedure has done on three consecutive days on the same flower to ensure successful cross pollination and hybrid seed production. Hand pollinated flowers were allowed to remain in the plant itself for the next 2-3 weeks for the maturation and natural drying of the seeds. The seeds were separately collected from the dried flowers, shade dried and maintained at 4 ^oC.

3.1.3 Raising the F_1 population

The F_1 seeds were sown in portrays containing cocopeat, vermiculate and perlite in the ratio 3:1:1, under greenhouse conditions. A total of 30 hybrid plants were subsequently transplanted in pots maintained under greenhouse conditions and containing sterilized soil (Plate 2a and 2b). The plants were found to have the characters of both resistant and susceptible parents. Regular watering and timely top dressing were given.

The flower buds at the initiation of opening were covered with butter paper bags to avoid accidental cross pollinations (Plate 2c). The flowers were selfed by hand pollination with the pollen from the same flowers consecutively for three days. The selfed seeds were collected 3-4 weeks after covering, shade dried and maintained at 4 ^oC.

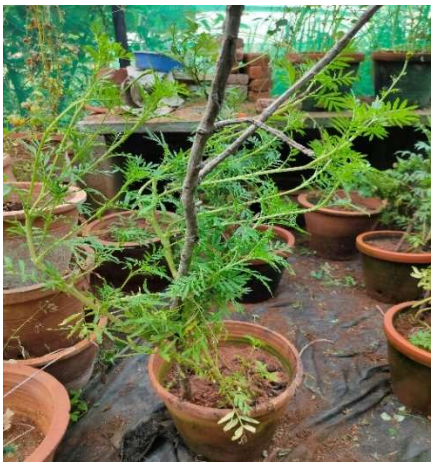
a. Protray nursery of resistant and susceptible parents

b. Resistant and susceptible plants in pots under greenhouse conditions

c. Flowers bagged for hand pollination

Plate 1. Raising of parental population

a. Raising of F_1 plants as pot culture



b. F_1 plant

c. Flowers of male and female parents bagged to avoid pollen contamination

Plate 2. Raising of F_1 plant population

3.1.4 Raising and screening the F_2 mapping population

The seeds obtained by selfing of F_1 plants were sown in portrays (Plate 3c).

Bacterial pathogen was isolated from diseased plants of cv. Double Yellow, at the peak of infection (Plate 3a). The diseased plants were washed thoroughly and cut it into several pieces for collecting the ooze (Plate 3b). The oozed-out inoculum was collected under lab conditions and root tips of the three week old F_2 seedlings were slightly trimmed off and incubated in the oozed-out solution for 10 minutes (Plate 4a and 4b). The ooze was further diluted to 10^8 cfu/ml with an OD value of 0.4 at 600 nm.

The seedlings were transplanted to four inch sized pots (Plate 4c) filled with sterilized cocopeat, vermiculite and perlite at 3:1:1 ratio and maintained under greenhouse conditions (Plate 5a and 5b). Twenty five ml of the freshly prepared ooze solution at 10^8 cfu/ml strength was also poured around each plant on the same day of transplantation. Parental plants were also maintained and screened along with the F_2 population.

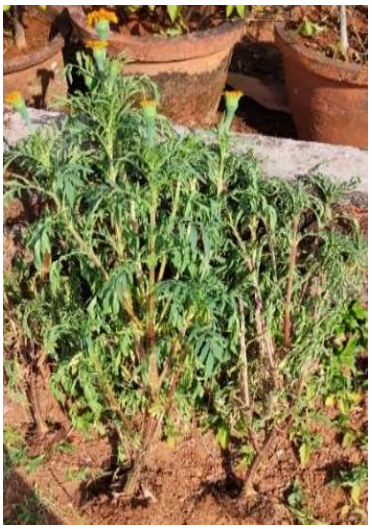
Two hundred and four segregating plants were maintained in pots and watered with 25 ml of distilled water per pot, every day. For BSA, leaf samples were collected from all the 204 plants before inoculation and maintained at -80 °C.

3.1.4 Evaluation of F_2 individuals

Every day after inoculation, plants were checked for the wilt symptoms. The incidence of the disease was recorded. Based on the intensity of the disease, highly susceptible, moderately susceptible, moderately resistant and highly resistant plants were identified from the F_2 population. The disease was confirmed again by ooze test under laboratory conditions. For ooze test, slant cut was given at the lower portion of the stem near the root, plants dipped into the beaker containing fresh water and held for five minutes. Infected plants had shown bacterial streaming from the cut end (Plate 6a and 6b). The picture of field after one month of inoculation has shown in Plate 6c.

3.2 Bulked Segregant Analysis (BSA)

BSA was performed using the DNA isolated and maintained from the F_2 plants. DNA from the identified most susceptible and resistant F2 plants were assayed using ISSR marker system and the markers segregating with the trait were identified.



a. Wilted plants collected to prepare bacterial culture

b. Oozing out of *Ralstoia solanacearum* for artificial inoculation

c. F_2 seedlings raised in protrays

Plate 3. Production of Fresh bacterial culture production and raising of F_2 seedlings

a. Root tip clipping for effective inoculation

b. Dipping the seedlings in fresh bacterial culture for 10 min

c. Transplanting of F_2 seedlings in pots

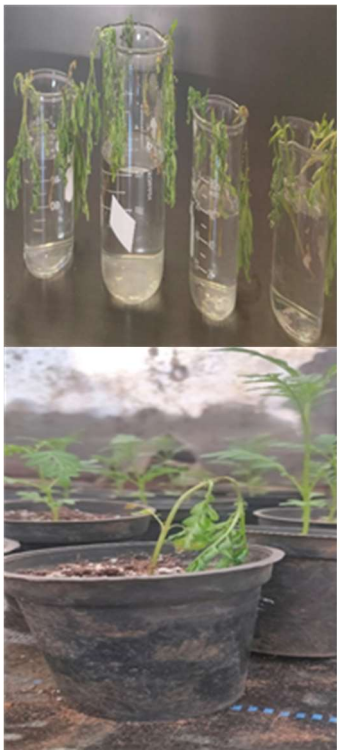
Plate 4. Artificial inoculation in F_2 seedlings

a. Raising of resistant and susceptible plants as disease checks

b. F_2 plants after transplanting into pots

c. Transplanting of F_2 seedlings in pots

Plate 5. Transplanting of F2 seedlings into pots



a. Susceptible F_2 plant showing wilt symptoms

b. Ooze out test conducted for bacterial wilt confirmation

c. Status of the seedlings after one month of inoculation

Plate 6. Ooze test for bacterial wilt in F_2 population

3.2.1 DNA isolation

Tender leaves collected at the early growth stages, from both parental and F_2 population, and maintained under -80 °C were used for DNA isolation. DNA was isolated from the tender leaves of both parents and 10 each of most susceptible and resistant F_2 plants, using CTAB method.

3.2.1.1 Reagents used

A. CTAB extraction buffer (2X)

- 2 per cent CTAB (w/v)
- 100 mM Tris Base (pH 8.0)
- 20 mM EDTA (pH 8.0)
- 1.4 M NaCl
- 1 per cent polyvinyl pyrrolidone (PVP)
- 0.2 per cent β-mercaptoethanol

B. Liquid nitrogen

C. Chloroform:Isoamyl alcohol (24:1 ratio v/v)

D. 70 per cent ethanol

E. 100 per cent ethanol

F. Ice cold Iso-propanol

G. RNase A (1 per cent)

3.2.1.2 Procedure

- CTAB extraction buffer (2X) taken in a 2 ml centrifuge tube was preheated to 65 ºC in a water bath
- One gram of tender leaf tissue was ground in pre-cooled pestle and mortar using liquid nitrogen and made into fine powder and mixed with pre-heated CTAB extraction buffer and a pinch of PVP
- The tubes were incubated in water bath at 65 °C for one hour, with occasional gentle inversions
- Tubes were kept on ice for 5-10 minutes after incubation and equal volume of chloroform-isoamyl alcohol (24:1) was added and mixed by gentle inversion

- The mixture was centrifuged at 10,000 rpm at room temperature for 15 minutes (Kubota 6500)
- The aqueous top layer solution was pipetted to fresh centrifuge tube
- 1.0 µL of RNase was pipetted into the tube and incubated at 37°C for 20 minutes
- Then the tubes were centrifuged at 13,000 rpm for 15 minutes. The supernatant was pipetted out into a 1.5 ml centrifuge tube
- The washing was repeated until a clear solution obtained
- One sixth volume of ice-cold isopropanol was added to the tube, mixed gently and incubated at -20 °C for one hour for complete precipitation of the DNA
- After incubation, the tubes were centrifuged at 4 ºC, 10,000 rpm for 15 minutes and the supernatant was carefully discarded without disturbing the pellet
- The pellet was washed by centrifuging with 200 µL of 100 per cent ethanol at 8,000 rpm for 2 minutes and repeating the washing using 100 µL of 70 per cent alcohol
- The pellet was air dried and dissolved completely in 50 µL of TE buffer and stored at -20 ºC

3.2.2 Quality assessment by agarose gel electrophoresis

Quality of the isolated DNA was assessed by electrophoresis on 0.8 per cent agarose gel (Sambrook *et al.,* 1989). Intact single band has shown the good quality of DNA (Plate 7 and 8).

3.2.2.1 Equipment

- Electrophoresis unit including the power pack, gel casting tray, comb
- Gel documentation system (BioRad^{TM} XR^{+})

3.2.2.2 Reagents

A. Agarose (Promega) 0.8 per cent (w/v)

B. Tris Acetic acid EDTA (TAE) buffer - 50X

- Tris base - 242 g

- Glacial acetic acid - 57.1 mL

- 0.5 mM EDTA - 100 mL

C. Loading dye (Bangalore Genei)- 6X

D. Ethidium bromide - stock concentration 10 mg mL^{-1}; working concentration 0.5µg mL^{-1}.

3.2.2.3 Procedure

- Agarose gel solution (0.8 per cent) was prepared by dissolving 0.48 g of agarose in 60 mL of 1X TAE. Agarose was heat melted to get a clear solution
- When the temperature of the solution becomes bearable, 5 µL of ethidium bromide (10 ml L^{-1}) was added and mixed thoroughly
- Casting tray (60 ml capacity) was fixed in the gel casting apparatus and the comb was placed at around one inch from the top of the tray
- Agarose gel solution was poured into the tray and left to solidify for about 30-40 minutes
- Once solidified, the casting apparatus was dismantled and the gel tray was placed inside the horizontal electrophoresis unit and the unit was filled with 1X TAE buffer solution, to a point where all the wells were submerged
- 4 µL of suitable DNA ladder was loaded into the first well
- 2 µL of 6X gel loading dye was mixed with 4 µL of sample DNA and loaded into the wells
- The tank was closed and anode and cathode were connected to respective points in the power pack. Electrophoresis was carried out by applying a constant voltage of 80 V and was allowed to run until the tracking dye reached two third of the length of the gel
- The gel was taken out from the unit and studied under Gel-Doc unit

3.2.2.4 Gel documentation

Gel documentation was done by carefully transferring the gel to the documentation system and exposing it to UV light. Images were optimized by clicking trans-UV and exposing with the software Quantity One. The presence of

protein was observed as a thick white patch restricted in the wells itself. The RNA contamination appeared as thick band in the region below 500 bp.

3.2.3 Assessment of DNA quality and quantity by spectrophotometer

The quantity and quality of the isolated DNA was estimated by spectrophotometer **(**NanoDrop® ND-1000). Maximum absorbance shown by nucleic acids and proteins are at 260 and 280 nm, respectively. The purity of DNA was checked by calculating the $OD_{260}/_{280}$ ratio. Values between 1.8 and 2.0 indicated good quality DNA with negligible amount of RNA and protein contamination. Values below 1.8 indicated protein contamination while above 2.0 indicate RNA contamination.

3.2.3.1 Procedure

- The system was connected to the NanoDrop spectrophotometer and software ND-1000 was opened
- The nucleic acid option was selected, sampling arm lifted and the upper and lower measurement pedestals wiped carefully.
- 1.0 µL of distilled water was pipetted onto the lower measurement pedestal. Sampling arm was closed and spectral measurement was initiated in the software
- The absorption reading was set to zero with blank sample
- After wiping both the pedestals, 1.0 µL of DNA sample was added into the lower pedestal and the option measure was selected
- Upon completion of measurement, upper and lower pedestals were thoroughly wiped using a soft laboratory wipe and the next sample was measured

3.2.4 Bulking of DNA samples

For BSA, 10 DNA samples each from the highly resistant and highly susceptible plants from the F_2 population (Plate 9 and 10) were bulked separately. The resistant and susceptible DNA bulks were diluted to 20 ng μL^{-1} and used for ISSR analysis.

3.2.5 Thermal cycling

The PCR reaction mixture (20 µL) had

a) Genomic DNA (20ng/µL) - 1.5 µL

b) 10X *Taq* assay buffer A (GeNie) - 2.0 µL

c) dNTPs mix (10 mM each) (GeNie) - 1.8 µL

d) *Taq* DNA polymerase (3U) (GeNie)- 0.4 µL

e) Primer (10 pM) (Sigma Aldrich) - 2.5 µL

f) Autoclaved distilled water - 11.8 µL

Total volume - 20.0 µL

The thermal profile used in the thermal cycling was,

Initial denaturation – 95 °C for 2 minutes

Denaturation – 95 °C for 30 seconds

Primer annealing – desired temperature for 45 seconds } 40 cycles

Primer extension – 72 °C for 2 minutes

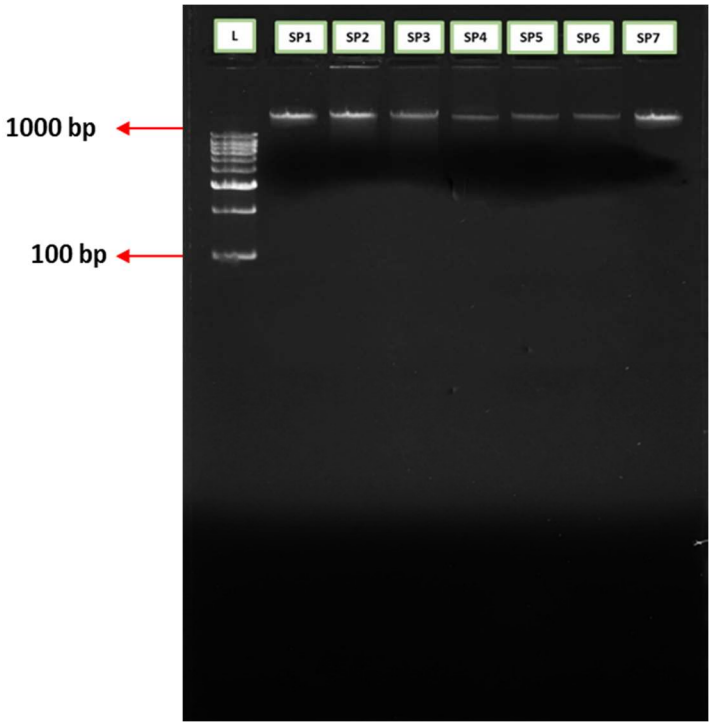
Final extension – 72 °C for 10 minutes

20 µL of the PCR product was mixed with 3 ml of 6X gel loading dye and electrophoresed on 1.8 per cent agarose gel at 80V until the tracking dye had reached almost 3/4th of the gel. Amplification pattern in resistant and susceptible parents and the resistant and susceptible bulks was checked in gel documentation system.

L : 100 bp Molecular weight ladder

RP1 – RP7 : Resistant parent 1 to 7

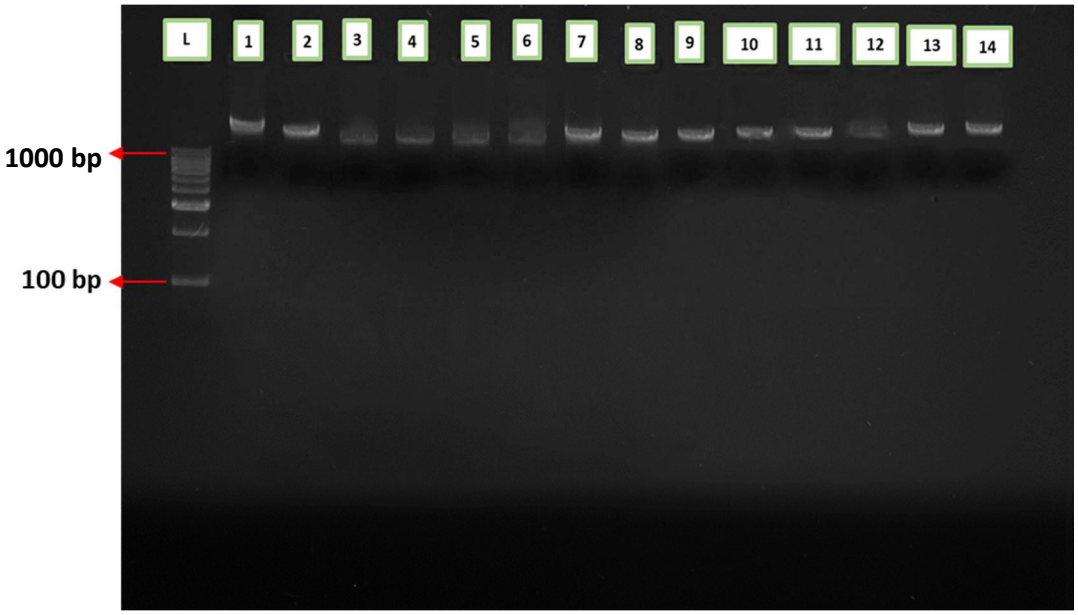
Plate 7: Agarose gel image of DNA isolated from resistant parent 1 to resistant parent 7



L : 100 bp Molecular weight ladder

RP1 – RP7 : Susceptible parent 1 to 7

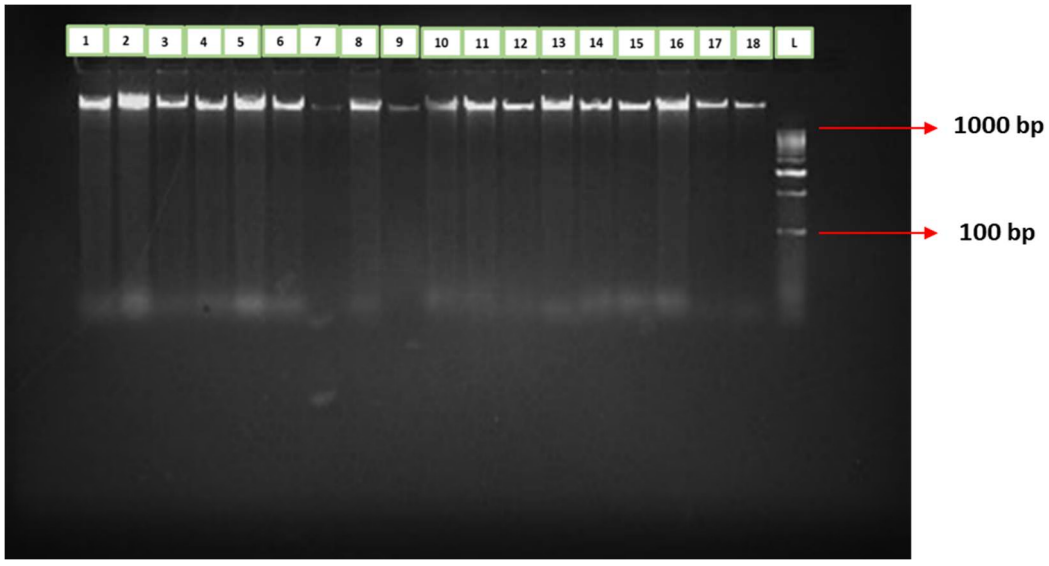
Plate 8: Agarose gel image of DNA isolated from susceptible parent 1 to susceptible parent



L : 100 bp Molecular weight ladder

1 -14 : F_2 resistant plant 1 to F_2 resistant plant 14

Plate 9: Agarose gel image of DNA isolated from F_2 resistant plant 1 to resistant plant 14



L : 100 bp Molecular weight ladder

1 -14 : F_2 susceptible plant 1 to F_2 susceptible plant 14

Plate 10: Agarose gel image of DNA isolated from F_2 susceptible plant 1 to susceptible plant 14

3.2.6 Screening of primers

BSA was done using selected ISSR primers (Table 3.1).

Using each primer, DNA from the resistant parent, susceptible parent, resistant F_2 bulk and susceptible F_2 bulk were amplified and checked for the marker polymorphism.

Of the 50 primers tested, 21 showed good amplification pattern and they were used for BSA.

Table 3.1. Primers used in BSA

Sl. No.	Primer	Primer sequence
1	UBC 811	5′ AGAGAGAGAGAGAGAC 3′
2	ISSR 31	5′ AGAGAGAGAGAGAGAGVC 3′
3	ISSR 22	5′ TCTCTCTCTCTCTCTCC 3′
4	ISSR 34	5′ AGAGAGAGAGAGAGAGAA 3′
5	UBC 857	5′ ACACACACACACACACYG 3′
6	ISSR 16	5′ AGAGAGAGAGAGAGAGG 3′
7	UBS 866	5′ CTCCTCCTCCTCCTCCTC 3′
8	ISSR 30	5′ CTCCTCCTCCTCRC 3′
9	ISSR 03	5′ GAGAGAGAGAGAGAGAYT 3′
10	UBC 888	5′ BDBCACACACACACACA 3′
11	ISSR 06	5′ AGAGAGAGAGAGAGAGYT 3′
12	UBC 823	5′ CACACACACACACACAG 3′
13	ISSR 17	5′-GAGAGAGAGAGAGAGAT-3′
14	UBC 847	5′ - CACACACACACACACARC 3′
15	ISSR 12	5′AGAGAGAGAGAGAGAGC 3′

16	ISSR 18	5′ GAGAGAGAGAGAGAGAC 3′
17	ISSR 1	5′ CACACACACACACACARG 3′
18	ISSR 20	5′ GTGTGTGTGTGTGTGTC 3′
19	ISSR 21	5′ TCTCTCTCTCTCTCTCG 3′
20	UBC 817	5′ CACACACACACACACAA 3′
21	ISSR 4	5′ CTCTCTCTCTCTCTCTG 3′

3.2.7 Marker for bacterial wilt resistance in marigold

The banding pattern generated using each primer were carefully analyzed to identify the bands present in the resistant parent as well as absent in the resistant F_2 bulk and missing in susceptible parent and susceptible F_2 bulk or the vice versa. Such bands were designated as the markers segregating with the trait. Marker identified in the susceptible samples showed that the resistance is governed by the recessive gene.

Results and Discussion

4. RESULTS AND DISCUSSION

Results of the study "Identification of molecular marker linked with bacterial wilt resistance in marigold (*Tagetes erecta* L.)" carried out at Centre for Plant Biotechnology and Molecular Biology, College of Agriculture, KAU, during 2019-21, are presented and discussed in this chapter. This study was done with the objective to identify a molecular marker associated with bacterial resistance in marigold, using bulk segregant analysis, to assist in the development of bacterial wilt resistant varieties, which is the only practical method to mitigate the losses caused by this soil borne bacterium (Nimisha, 2016).

4.1 Field screening for bacterial wilt incidence

Generating a mapping population segregating for the trait is the prerequisite in BSA for marker development. An F_2 population was developed by crossing the resistant and susceptible genotypes.

4.1.1 Screening the parents

The parents were raised in pots under greenhouse conditions. Resistant parent M_1 has shown 100 per cent resistance after artificial inoculation. The susceptible parent Double Yellow showed disease symptoms, three weeks after planting.

Flower buds of cv. Double Yellow as well as the male parents were bagged when these just initiated opening. Upon opening, the successive outer ray florets of the female parent were hand pollinated with the pollen from M_1 and M_2. Among two varieties, one local accession and five F_1 hybrids evaluated at KAU, the local accession M_1 was found to be resistant and 100 per cent wilting was shown by the F_1 hybrid Sakura 031. But M_1 plants were having low quality flowers and poor yield (0.234 kg/plant) (Umesh *et al*., 2018). So, there arise a need for developing plants having good quality flowers and higher yield with bacterial wilt resistance.

4.1.2 Disease response in F_1 population

Since the genetics of bacterial wilt resistance in marigold is not known, the F_1 plants were not screened artificially. The resistance if governed by the susceptible

genes, shall lead to the mortality of the population under artificial screening. Thirty F_1 plants raised in pots under greenhouse conditions have shown moderate resistance. Plants showed disease symptoms three months after planting, at flowering period. This suggested that the resistance is polygenic.

Flowers in F_1 plants were bagged and selfed, butter paper bags retained to avoid pollen contamination and seeds were collected.

4.1.3 Raising and screening the F_2 population

Fresh bacterial ooze with OD value above 0.9 was used to screen the F_2 population, by dipping the plants for 10 minutes before planting. Complete infection was obtained by this inoculation process. Umesh *et al.* (2018) studied the methods such as media drenching, root dip and stem injection for artificial inoculation in marigold and reported all the methods have the same level of efficiency for developing the infection.

Since the fresh culture had virulent pathogenic population, it was highly effective than the cultured medium (Serizawa *et al*., 1989). Jeevan *et al.* (2020) used the same method for inoculation of bacterial wilt in marigold where he added an inoculum of concentration 3.01 x 10^5 cfu/ ml and the symptoms started to appear in six days after inoculation.

The disease severity in these plants were high and plants started to wilt from the third day of planting. This result was in accordance with the results reported by Jeevan *et al*. (2020), where African marigold genotypes were assessed for resistance. During infection, the susceptible plants showed symptoms like yellowing of leaves, stunted growth, shedding and drying of flowers and finally the plant mortality. The same symptoms appeared in a study where wilting started appearing from the lower leaves, spreads all over and caused stunted growth. At later stages, recovery was impossible and plants died within 3-4 days (Walker, 1952).

Of the 204 F_2 plants, 140 plants showed different levels of susceptibility to bacterial wilt and 64 plants were resistant. The plants were precisely divided into highly susceptible, moderately susceptible, moderately resistant and highly resistant according to the period for occurrence of symptoms. The severity of the disease

incidence in the genotypes was scored as per the scoring followed by Sinha *et al*. (1988) (Table 4.1).

Table 4.1. Levels of resistance seen in F_2 mapping population

Disease response	Number of plants
Highly susceptible	40
Moderately susceptible	100
Moderately resistant	34
Highly resistant	30

4.1.4. Time of wilting in F_2 population

The F_2 plants started to wilt in three days after inoculation and transplanting. The infection in all the wilted plants was confirmed by using ooze out test. Plants which were healthy during first few days showed the symptoms subsequently and it has been recorded on daily basis. The frequency of wilting shown by the F_2 seedlings is depicted in Fig.1

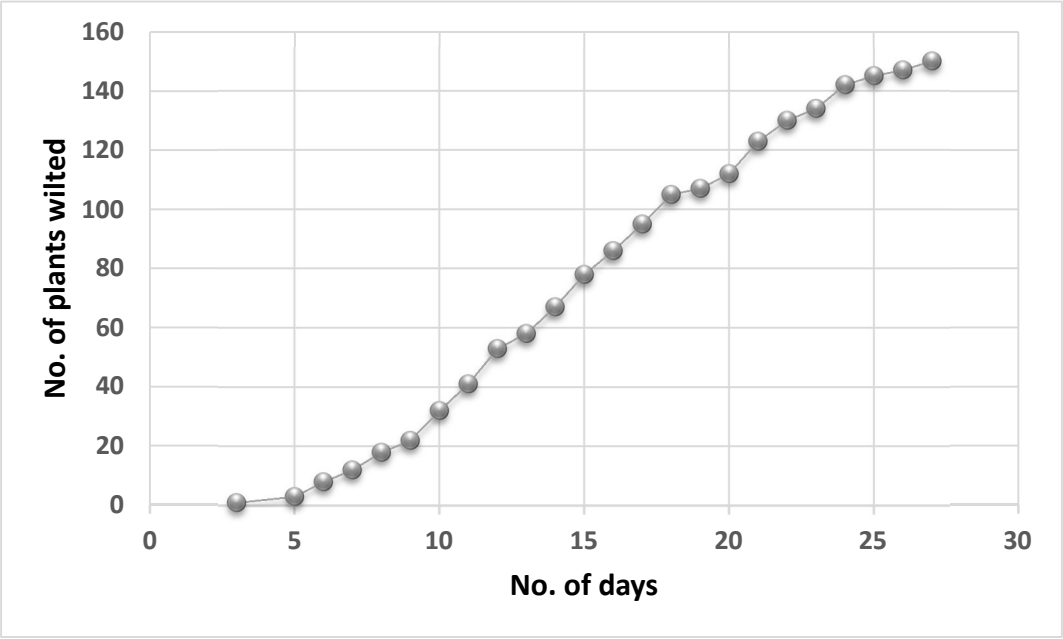


Fig 1. Wilting frequency of F_2 seedlings after artificial inoculation

4.2 Bulked Segregant Analysis

BSA on the mapping population segregating for resistance was done with ISSR marker analysis for identifying the markers linked with bacterial wilt resistance gene. Random marker systems have been reported to be easier to use in BSA since the number of bands generated will be more.

Ten plants each from resistant and susceptible groups were bulked in BSA. The probability of an unlinked locus being polymorphic between two bulks of 10 individuals was calculated to be 2×10^{-6} (Michelmore *et al*., 1991), for individuals coming from genetically diverse pools, single segregating population or varietal mixtures (Virk *et al*., 1996).

4.2.1 Isolation of DNA

Among the DNA isolation protocols attempted, highest quantity of DNA was obtained from modified CTAB and Dellaporta methods. Among them, modified 2 per cent CTAB method was better. Good quality DNA with no RNA and protein contamination was obtained, which is shown in Plate 7,8,9 and10.

In a study by Shahzadi *et al.* (2010), isoamylalcohol, CTAB, and SDS methods were compared for DNA isolation from marigold. CTAB method proved to be the most efficient because DNA from other two methods contained high levels of polysaccharides, flavonoids, polyphenols and essential oils. This affects the polymerase activity and hinders DNA amplification.

4.2.2 Quantity and quality assessment of DNA

Isolated DNA were electrophoresed on 0.8 per cent agarose gel by staining with Ethidium Bromide (EtBr) using 1x TAE buffer. Wettasinghe and Peffley (1998) reported that if sample had an intact band of high molecular weight, with very minimum smearing and minimum contamination by RNA, it is of good quality. The samples isolated in the study showed intact high molecular weight bands with very less smearing. Minor RNA contamination was cleared by RNase treatment. The gel image of the isolated DNA from the resistant parent, susceptible parent, resistant F_2, susceptible F_2 are shown in Plate 7,8,9,10.

The samples showing good quality DNA on electrophoresis were analyzed for quality and quantity using spectrophotometer. DNA having the $A_{260/280}$ ratio within the range of 1.8 and 2.0 were selected for bulking (Meena, 2014; Thakur *et al*., 2014). The spectrometer reading of parental and F_2 DNAs are presented in Table 4.2. The average quantity and $A_{260/280}$ ratio for M_1 DNA was 1124.6 and 1.84 respectively. Similarly, average quantity and $A_{260/280}$ ratio for Double Yellow DNA were 1733.58 and 1.88.

Table 4.2 Quantity and quality of DNA isolated from the F_2 plants

Sl. No.	Name	Quantity (ng/µL)	$A_{260/280}$
1	RF_2	2256.5	1.85
2	RF_2	1299	1.82
3	RF_2	1279	1.83
4	RF_2	743	1.8
5	RF_2	1680.5	1.9
6	RF_2	1638.5	1.8
7	RF_2	788.5	1.87
8	RF_2	1259.5	1.9
9	RF_2	965	1.92
10	RF_2	1156	1.86
11	RF_2	789	1.82
12	SF_2	1775	1.75
13	SF_2	890	1.8
14	SF_2	2905	2
15	SF_2	1154	1.83
16	SF_2	3745	1.7
17	SF_2	1052.3	1.83
18	SF_2	943.55	1.8
19	SF_2	253.35	1.9
20	SF_2	657	1.8

4.2.3 Pooling of DNA samples for BSA

For primer screening in BSA, 10 DNA samples from each of the highly resistant and highly susceptible plants were taken and bulked separately. Usually, F_2 population has been used for BSA because it provides the best recombination and segregation in the population so that extreme phenotypes can be easily distinguished upon artificial inoculation. F_2 population got the capacity to generate a much larger segregating population when compared to DH lines and NILs (Michelmore *et al*., 1991).

Quantitative trait locus mapping requires a mapping population, each member of which has to be genotyped with the molecular markers selected to cover the genome, and phenotyped for the traits of interest. Genotyping a large mapping population is tedious and relatively costly. By grouping plants according to the extreme expressions for a particular trait and extracting DNA from these two bulks, the process of genotyping the plants can be reduced to two DNA samples. This strategy called BSA, was first described by Michelmore *et al.* (1991) who studied the disease resistance in lettuce.

BSA has been efficiently employed in molecular marker finding for important traits by He *et al*. (2009), Bi-Hao *et al*. (2009), Ragina and Sadhankumar (2013), Yanping *et al*. (2013), Thakur *et al*. (2014), Hui *et al*. (2019) and many others. In BSA, plants from segregating populations are grouped according to the phenotypical expressions and extreme phenotypes of the targeted trait shall be selected and used for checking the allele differences among the population bulk. Hence, we can access the marker associated with the trait without forming a map (Quarrie *et al*., 1999; Magwene *et al*., 2011). In the study conducted by Michelmore *et al*. (1991), 14-15 plants from both category were selected to form the bulks and analyzed for the DNA polymorphisms linked with the disease resistance in lettuce, using RAPD marker system. Though BSA is a short cut in marker finding, many a times the polymorphism obtained is reported to be low (He *et al.*, 2009).

Bulk Segregant Analysis was conducted for the validation of SCAR4 marker linked with male sterility in marigold. F_2 population with 164 male fertile and 62 male sterile plants was developed by crossing the male sterile line IIHR10521AB and

inbred line IIHRMY7. The plants were screened against SCAR4 marker and its linkage with the sterility locus was confirmed (Asha *et al*., 2019).

Bacterial wilt resistance may be governed by polygenes or oligogenes. Resistance controlled by polygenes show moderate resistance whereas highly resistant lines will have both oligo and polygenes (Nishi *et al*., 2003).

4.2.4 Optimization of thermal amplification

Ten DNAs each from resistant and susceptible F_2 plants were bulked to form the resistant bulk and susceptible bulk, respectively. Yaping and Daocheng (2013) had reported that 25 ngμL^{-1} concentration of marigold DNA is optimum for ISSR PCR reactions. Hence, the concentrations of the resistant and susceptible bulk DNA were brought down to 25 ngμL^{-1} for polymerase chain reactions.

The annealing temperature plays a central role in a PCR reaction, and it is theoretically 5 ºC lesser than the melting temperature of the primer. However, the optimum experimental value slightly varies and needs to be optimized (Rychlik *et al*., 1990). Gradient PCR, a variant of normal PCR, was used to standardize the annealing temperature of each primer. For each primer, gradient PCR at a temperature range of ±5 ºC of the theoretical annealing temperature was run. The banding pattern and annealing temperature of the primers are given in Table 4.3. The annealing temperature of most of the primer was in between 48 to 52 °C (Liu *et al*., 2013). The temperature at which maximum number of clear bands were amplified was achieved was selected as the optimum.

Table 4. 3. Sequences and annealing temperatures of the ISSR primers

Sl. No.	Primer	Nucleotide Sequence	Annealing Temperature (°C)
1	UBC 811	5′ AGAGAGAGAGAGAGAC 3′	50
2	ISSR 31	5′ AGAGAGAGAGAGAGAGVC 3′	53.5
3	ISSR 22	5′ TCTCTCTCTCTCTCTCC 3′	52
4	ISSR 34	5′ AGAGAGAGAGAGAGAGAA 3′	49
5	UBC 857	5′ ACACACACACACACACYG 3′	57

6	ISSR 16	5′ AGAGAGAGAGAGAGAGG 3′	48
7	UBS 866	5′ CTCCTCCTCCTCCTCCTC 3′	52
8	ISSR 30	5′ CTCCTCCTCCTCRC 3′	52
9	ISSR 03	5′ GAGAGAGAGAGAGAGAYT 3′	50
10	UBC 888	5′ BDBCACACACACACACA 3′	48
11	ISSR 06	5′ AGAGAGAGAGAGAGAGYT 3′	50
12	UBC 823	5′ CACACACACACACACAG 3′	50
13	ISSR 17	5′-GAGAGAGAGAGAGAGAT-3′	52
14	UBC 847	5′ - CACACACACACACACARC 3′	50
15	ISSR 12	5′AGAGAGAGAGAGAGAGC 3′	52
16	ISSR 18	5′ GAGAGAGAGAGAGAGAC 3′	50
17	ISSR 1	5′ CACACACACACACACARG 3′	52
18	ISSR 20	5′ GTGTGTGTGTGTGTGTC 3′	52
19	ISSR 21	5′ TCTCTCTCTCTCTCTCG 3′	54
20	UBC 817	5′ CACACACACACACACAA 3′	52
21	ISSR 4	5′ CTCTCTCTCTCTCTCTG 3′	51

4.2.5 Amplification pattern using ISSR primers

DNA samples of resistant parent, susceptible parent, resistant F_2 bulk and susceptible F_2 bulk were screened with ISSR marker system. A 100-bp DNA ladder was used to analyze the size of each band.

Out of 21 primers analyzed, three have generated polymorphic bands associated with bacterial wilt resistance (Table 4.4).

Table 4.4. Amplification pattern in BSA with 21 ISSR primers (M_1 – resistant parent, S- susceptible parent, RF_2 – resistant F_2 bulk, SF_2 – susceptible F_2 bulk)

Sl. No.	Primer	Number of bands				Polymorphism	
		M_1	S	RF_2	SF_2	Parental	Trait linked
1	UBC 811	9	10	10	8	Polymorphic (1)	
2	ISSR 31	6	6	5	6	Monomorphic	
3	ISSR 22	9	8	8	9	Polymorphic (1)	
4	ISSR 34	7	8	8	8	Monomorphic	

5	UBC 857	4	4	4	4	Monomorphic	
6	ISSR 16	7	5	7	6	Polymorphic (3)	Linked (1)
7	UBS 866	8	9	9	9	Polymorphic (2)	Linked (2)
8	ISSR 30	13	11	13	12	Polymorphic (4)	Linked (2)
9	ISSR 03	6	6	6	6	Monomorphic	
10	UBC 888	6	6	6	6	Monomorphic	
11	ISSR 06	7	6	7	7	Monomorphic	
12	UBC 823	7	7	7	7	Monomorphic	
13	ISSR 17	8	8	8	8	Monomorphic	
14	UBC 847	9	8	8	9	Polymorphic (1)	
15	ISSR 12	11	8	10	10	Polymorphic (3)	Linked (1)
16	ISSR 18	8	8	9	9	Polymorphic (3)	
17	ISSR 1	9	9	9	9	Monomorphic	
18	ISSR 20	9	11	10	11	Polymorphic (1)	
19	ISSR 21	11	10	9	10	Polymorphic (3)	
20	UBC 817	12	13	10	10	Polymorphic (1)	
21	ISSR 4	11	10	10	11	Polymorphic (1)	

UBC 811

The ISSR primer UBC 811 had generated 9, 10, 10, 8 bands in resistant parent, susceptible parent, resistant F_2 bulk, susceptible F_2 bulk respectively. The sizes of the amplicons ranged at 250-1300 bp. An amplicon range of 300 bp showed parental polymorphism (bands present in susceptible parent and two bulks) but not associated with bacterial wilt resistance (Plate 11).

ISSR 31

Primer ISSR 31 generated 6, 6, 5, 6 amplicons in resistant parent, susceptible parent, resistant F_2 susceptible F_2, respectively. The amplicons ranged at 300-700 bp in size but all were monomorphic (Plate 11).

ISSR 22

ISSR 22 resulted 9, 8, 8 and 9 amplicons in resistant parent, susceptible parent, resistant F_2, susceptible F_2, respectively. Amplicons of 165-1450 bp were obtained and a parent polymorphic band was seen at 290 bp (Plate 12).

ISSR 34

Primer ISSR 34 produced 7, 8, 8 and 8 amplicons in resistant parent, susceptible parent, resistant F_2 and susceptible F_2, respectively. Band sizes ranged at 210-720 bp. and all were monomorphic (Plate 12).

UBC 857

UBC 857 has generated four bands each in all the samples, at 260-630 bp. All bands were monomorphic (Plate 12).

ISSR 16

ISSR 16 has generated 7, 5, 7 and 6 amplicons in resistant parent, susceptible parent, resistant F_2 and susceptible F_2, respectively. Band sizes ranged at 210-750 bp. Parental polymorphism was seen in the band at 610 bp (band present resistant parent and F_2 bulks). This primer has generated distinctly polymorphic band for resistance to bacterial wilt at 370 bp. This was present in both resistant parent and resistant F_2 population and absent in susceptible populations. So ISSR 16 can be used as a marker linked to bacterial wilt resistance (Plate 12).

UBC 866

ISSR analysis with primer UBC 866 has generated 8, 9, 9 and 9 amplicons respectively in resistant parent, susceptible parent, resistant F_2 and susceptible F_2 samples. The band sizes ranged at 220-700 bp. Two parental polymorphisms were obtained in a molecular size of 400 (bands present in susceptible parent) and 380 bp (bands present in resistant parent). These two bands were able to differentiate between F_2 bulks. So, these two polymorphic markers can be considered as molecular markers linked with bacterial wilt resistance (Plate 13).

ISSR 30

Primer ISSR 30 has produced 13, 11, 13 and 12 amplicons in resistant parent, susceptible parent, resistant F_2, susceptible F_2, respectively. Molecular weight of the bands ranged at 150-830 bp and parental polymorphism was seen at 780, 310 (bands present in resistant parent and two bulks), 150 bp and 800 bp sizes. The polymorphic bands at 150 bp was present in resistant parent and resistant bulk whereas the polymorphic band at 800 bp was present in susceptible parent and susceptible bulk. These markers are linked to bacterial wilt resistance (Plate 13).

ISSR 03

Primer ISSR 03 has produced six bands each in all the samples and the sizes ranged at 115-890 bp. All the bands were monomorphic (Plate 14).

UBC 888

This primer has generated six amplicons each in all the samples. The molecular weight of the monomorphic bands ranged at 90-530 bp (Plate 14).

ISSR 06

This primer has generated 7, 6, 7 and 7 amplicons in resistant parent, susceptible parent, resistant F_2, susceptible F_2, respectively and the sizes varied at 110-1500 bp. All the bands were monomorphic (Plate 14).

UBC 823

UBC 823 has generated seven monomorphic amplicons each in all the samples and their sizes ranged at 115-560 bp (Plate 14).

ISSR 17

ISSR 17 has generated eight monomorphic bands each in the samples, with their sizes ranging at 220-790 bp (Plate 15).

UBC 847

UBC 847 has generated 9, 8, 8 and 9 amplicons in resistant parent, susceptible parent, resistant F_2, susceptible F_2, respectively. Sizes of the amplicons ranged at 230-920 bp. Parental polymorphism was obtained at 600 bp (Plate 15).

ISSR 12

ISSR 12 has generated 11, 8, 10 and 10 amplicons in resistant parent, susceptible parent, resistant F_2 and susceptible F_2, respectively. Molecular weight of the bands ranged at 380-950 bp and parental polymorphism was seen at 600 and 680 bp (bands present in resistant parent and absent in susceptible parent). At 750 bp, marker linked with resistance was seen as it was present in resistant samples and absent in susceptible samples (Plate 15).

ISSR 18

ISSR 18 has generated 8, 8, 9 and 9 amplicons in resistant parent, susceptible parent, resistant F_2, susceptible F_2, respectively. Sizes of the bands ranged at 380-1400 bp with parental polymorphism seen at 690 bp (bands present in susceptible parent and two bulks) and at 500 and 1400 bp (bands present in resistant parent and two bulks) (Plate 16).

ISSR 1

This primer has generated nine bands in all the samples. Sizes of the amplicons ranged at 220-1100 bp and all were monomorphic (Plate 16).

ISSR 20

ISSR 20 primer has generated 9, 11, 10 and 11 amplicons in resistant parent, susceptible parent, resistant F_2 and susceptible F_2 samples, respectively. Sizes of the amplicons ranged at 210-1150 bp and parental polymorphism was found at 950 bp (band present in susceptible parent) (Plate 17).

ISSR 21

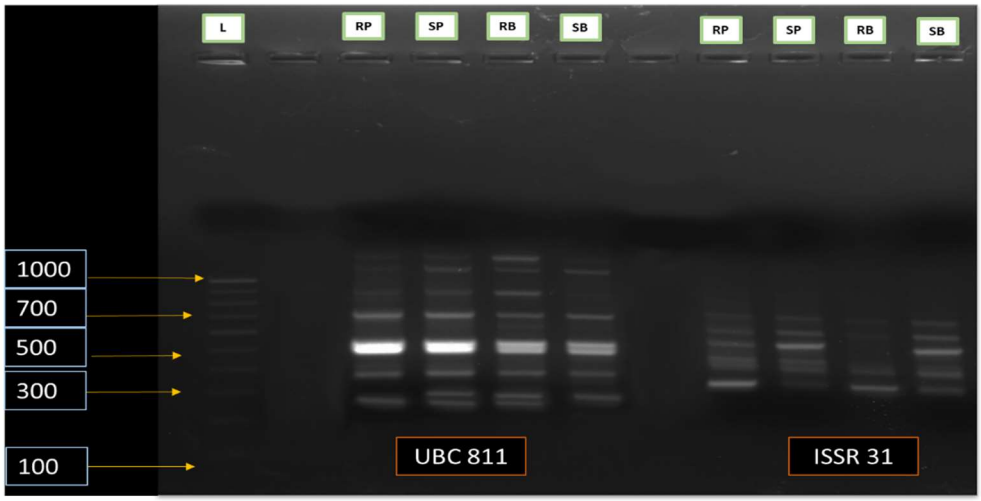
This primer produced 11, 10, 9 and 12 bands in the samples resistant parent, susceptible parent, resistant F_2 and susceptible F_2 samples, respectively. Sizes of the amplicons ranged at 200-880 bp and parental polymorphisms were found at 220 bp (bands present in resistant parent and susceptible bulk), 480 bp (bands present in resistant parent and two bulks) and 500 bp (bands present in susceptible parent and two bulks) (Plate 17).

UBC 817

UBC 817 has generated 12, 13, 10 and 10 bands in resistant parent, susceptible parent, resistant F_2 and susceptible F_2 samples, respectively. Sizes of the amplicons ranged at 490-1500 bp and parental polymorphism was seen at 705 bp (bands present in susceptible parent and two bulks) (Plate 18).

ISSR 4

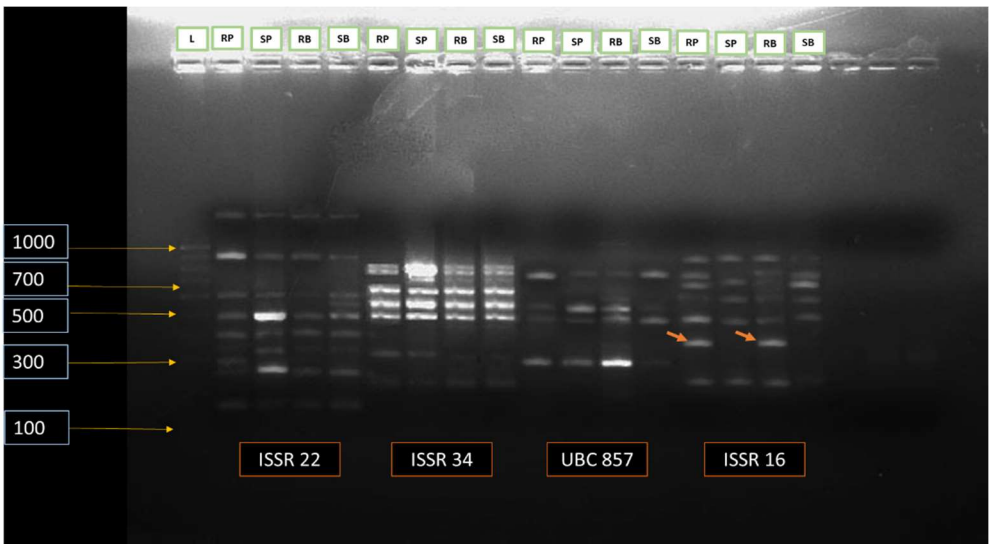
Primer ISSR 4 produced 11, 10, 10 and 11 bands in resistant parent, susceptible parent, resistant F_2 and susceptible F_2 samples, respectively. Sizes of the bands ranged at 375-870 bp and parental polymorphism was seen at 860 bp (band present only in susceptible parent) (Plate 19).



L – 100 bp ladder

RP – Resistant parent
SP – Susceptible parent
RB – F_2 resistant plants bulk
SB - F_2 susceptible plants bulk

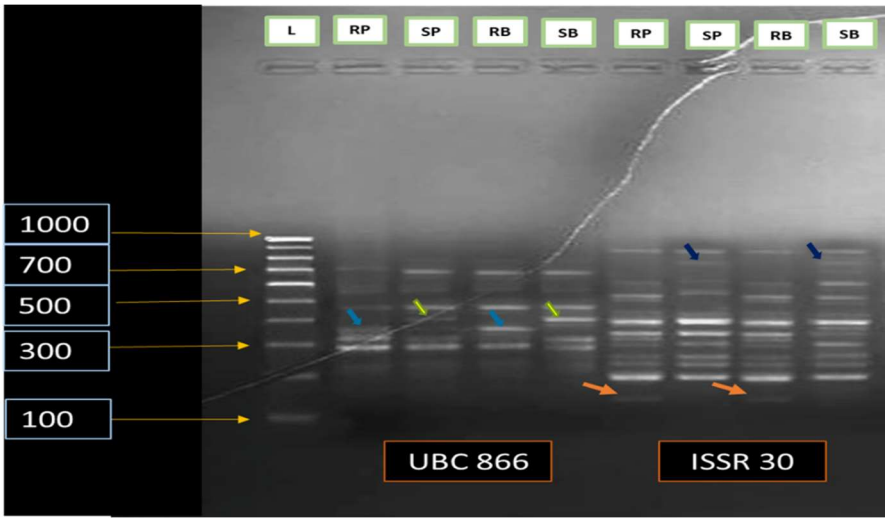
Plate 11: Amplification profile of UBC 811 and ISSR 31 primers



L – 100 bp ladder

RP – Resistant parent
SP – Susceptible parent
RB – F_2 resistant plants bulk
SB - F_2 susceptible plants bulk

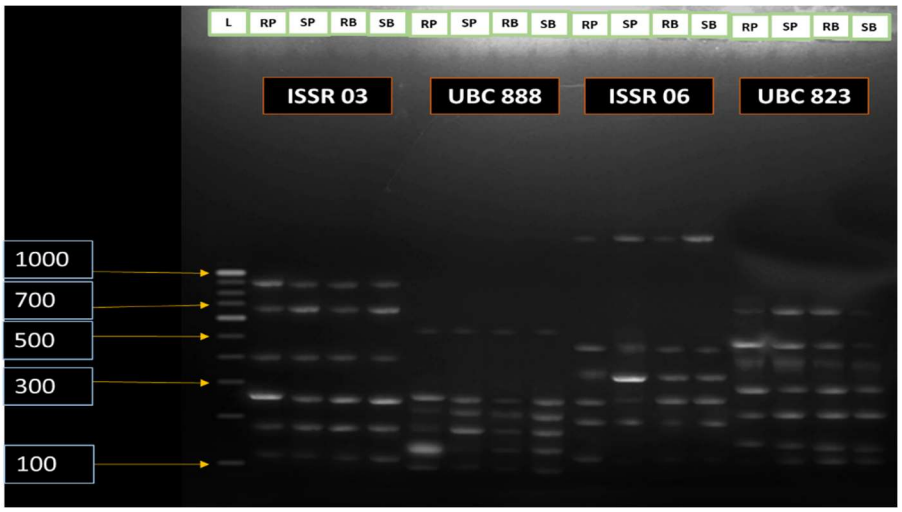
Plate 12: Amplification profile of ISSR 22, ISSR 34, UBC 857 and ISSR 16 primers



L – 100 bp ladder

RP – Resistant parent
SP – Susceptible parent
RB – F_2 resistant plants bulk
SB - F_2 susceptible plants bulk

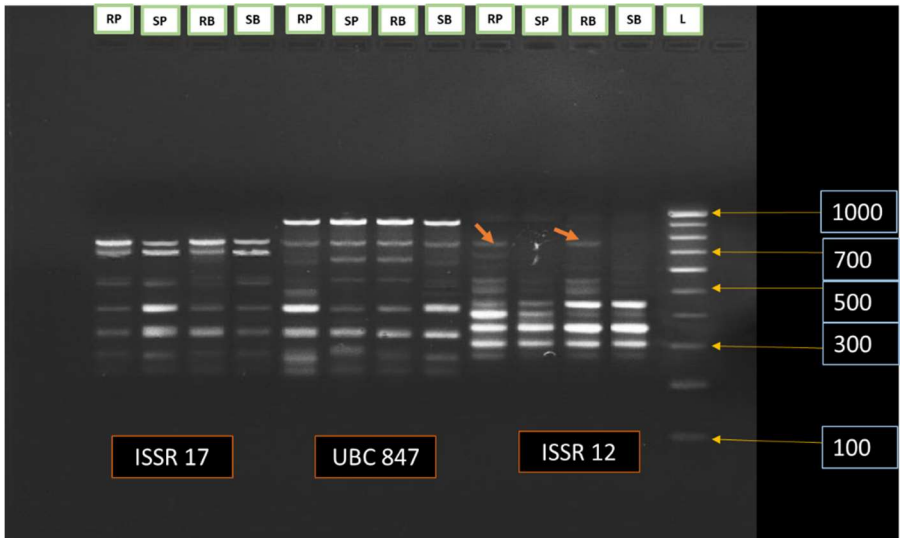
Plate 13: Amplification profile of UBC 836 and ISSR 30 primers



L – 100 bp ladder

RP – Resistant parent
SP – Susceptible parent
RB – F_2 resistant plants bulk
SB - F_2 susceptible plants bulk

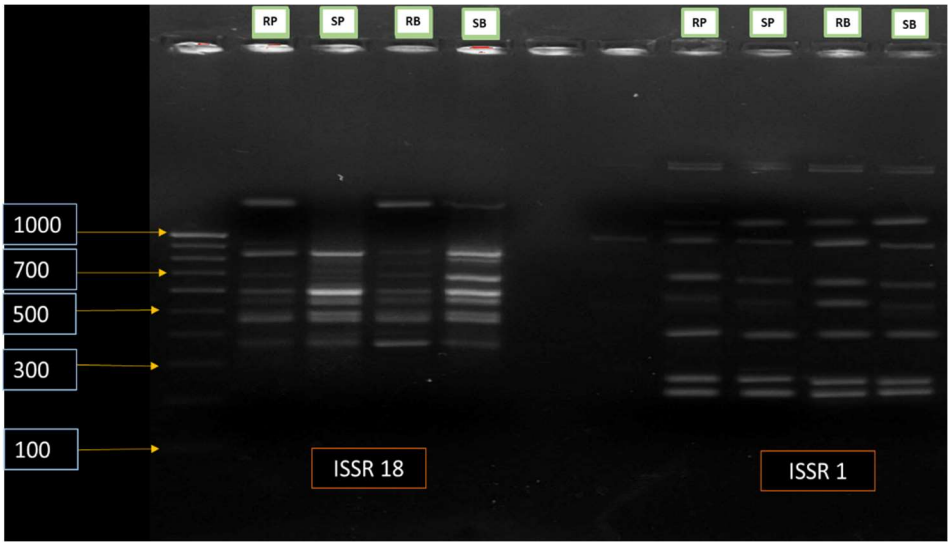
Plate 14: Amplification profile of ISSR 03, UBC 888, ISSR 06 and UBC 823 primers



L – 100 bp ladder

RP – Resistant parent
SP – Susceptible parent
RB – F_2 resistant plants bulk
SB - F_2 susceptible plants bulk

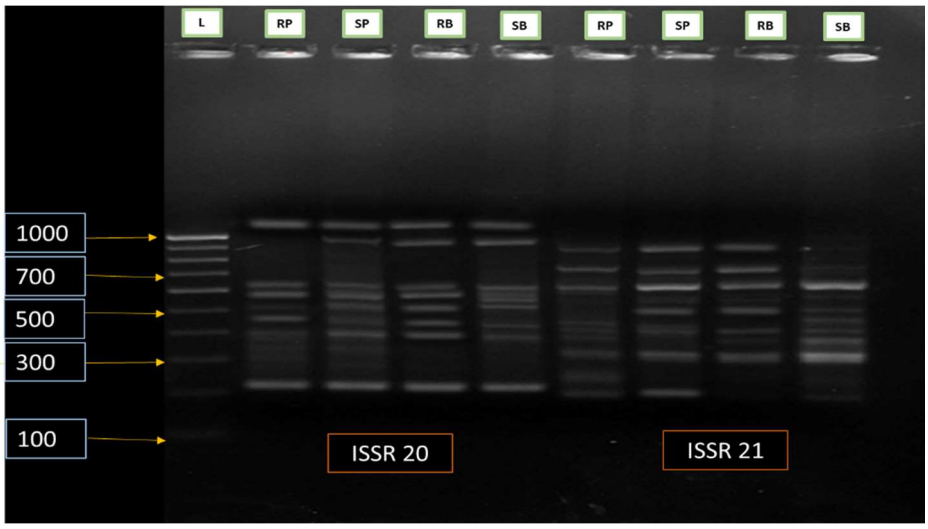
Plate 15: Amplification profile of ISSR 17, UBC 847 and ISSR 12 primers



L – 100 bp ladder

RP – Resistant parent
SP – Susceptible parent
RB – F_2 resistant plants bulk
SB - F_2 susceptible plants bulk

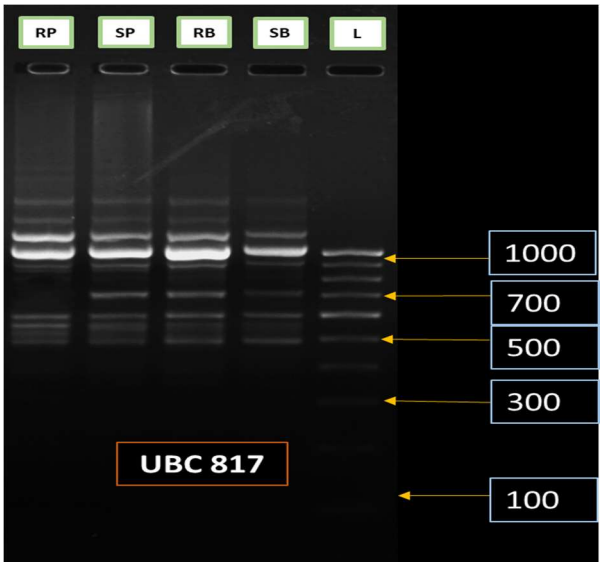
Plate 16: Amplification profile of ISSR 18 and ISSR 1 primers



L – 100 bp ladder

RP – Resistant parent
SP – Susceptible parent
RB – F_2 resistant plants bulk
SB - F_2 susceptible plants bulk

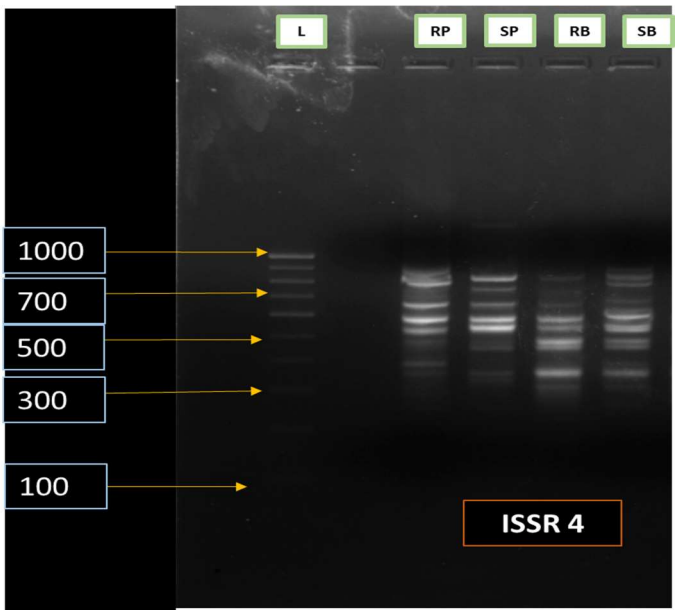
Plate 17: Amplification profile of ISSR 20 and ISSR 21 primers



L – 100 bp ladder

RP – Resistant parent
SP – Susceptible parent
RB – F_2 resistant plants bulk
SB - F_2 susceptible plants bulk

Plate 18: Amplification profile of UBC 817 primer



L – 100 bp ladder

RP – Resistant parent
SP – Susceptible parent
RB – F_2 resistant plants bulk
SB - F_2 susceptible plants bulk

Plate 19: Amplification profile of ISSR 4 primer

4.2.7 Marker analysis

The inter simple sequence repeat (ISSR) technique is a derivative of random amplified polymorphism DNA (RAPD) analysis but differs in that ISSR primer sequences are designed from microsatellite regions and the corresponding polymerase chain reaction annealing temperatures are generally higher than those applied to RAPD (Wolfe *et al*. 1998). In this experiment, 50 ISSR primers were tried to identify the DNA bands polymorphic between M_1 (Resistant parent) and Double Yellow (susceptible parent) and 21 were successful. A total of 179 bands were generated in both the parents from twenty-one primers, the remaining were faint or non-reproducible. Twenty-one polymorphic bands were obtained between the resistant and susceptible parents of which six were found to segregate with bacterial wilt resistance in marigold.

From this study, four ISSR markers at 750 bp in ISSR 12, 400 and 380 bp in UBC 866, 370 bp in ISSR 16 and 800 bp and 150 bp in ISSR 30 were identified for bacterial wilt resistance in marigold. In ISSR 12, three bands were polymorphic between parents and the one at 750 bp was linked with resistance. Similarly, study conducted by Namita *et al.* (2013) had shown that ISSR12 gives as high as 60 per cent polymorphism with a PIC value of 0.226, among different genotypes of marigold. The primer ISSR 16 is reported to generate high polymorphism and PIC value.

In case of ISSR 16, molecular marker associated with bacterial wilt resistance was found at 360 bp. The resistance was linked with resistance in both parental populations and bulks. Namita *et al.* (2013) also reported that ISSR 16 generated high polymorphism and PIC value in marigold.

Using ISSR 30, four parental polymorphisms were found and the marker at 150 bp segregated with resistance whereas markers at 800 bp segregated with susceptibility. The identification of a marker linked with susceptibility shows that at least one gene contributing to the resistance is recessive in nature. In marigold, primer ISSR 30 is reported to yield 89 per cent polymorphism and 0.24 PIC value (Panwar *et al*., 2018).

UBC 866 was found to be having two polymorphic markers associated with bacterial wilt resistance. The molecular sizes were found at 400 bp and 350 bp. The bands seen at 400 bp were associated with susceptibility while bands at 350 bp were associated with resistance. A study reported in genetic diversity analysis of Cucurbitaceae, UBC 866 was found to be having highest number of bands (24 bands) at a molecular range of 778 bp to 1968 bp (Sikdar *et al*., 2010). In a study conducted by Pandit *et al*. (2007), UBC 866 was found to be polymorphic in genetic diversity analysis of mango cultivars with 100 per cent of polymorphism.

Therefore, a total of six molecular markers linked to bacterial wilt resistance were identified. Four markers such as ISSR 12, ISSR 16, ISSR 30, UBC 866 were found to be linked with bacterial wilt resistance in marigold.

Use of ISSR markers in BSA helps to overcome several limitations that other markers are having such as low reproducibility of RAPD, high cost of AFLP and the need to know the flanking sequences to develop species specific primers as in case of microsatellite markers (Ratnaparkhe *et al.,* 1998; Reddy and Siddiq, 2002; Ajal *et al*., 2014; Duartea *et al*., 2018).

When 12 ISSR primers were used for diversity analysis in 15 genotypes of marigold, number of bands per primer ranged from 10 (ISSR-03, ISSR-07, ISSR-17, ISSR-29 and ISSR-31) to 15 (ISSR-02 and ISSR-12), at an average of 11.58. ISSR markers were reported to be having a greater number of bands and polymorphism content in marigold genotypes than RAPD markers. Upon cluster analysis of both African and French genotypes using ISSR markers, it was concluded that the variation among the genotypes of African marigold were less when compared to French genotypes (Namita *et al*., 2013). In contrast to this, Zeng *et al.* (2010) obtained lesser number of bands per primer (5 to 11, average 6.8) and 58.27 per cent were polymorphic, which was less than 138 polymorphic loci detected using 10 ISSR primers in *Tagetes patula* and *T. erecta*.

When SSR markers were designed in marigold based on ISSR markers, 34.38 per cent of SSRs were found polymorphic, compared to 25.51 per cent in the primers designed using SSR-enriched genomic library technique and 3.17 per cent in

transferable SSRs, indicating the efficiency of ISSR markers in marigold for polymorphic studies (Whankaew *et al*., 2014).

Genetic diversity of 22 marigold (*Tagetes erecta* L.) genotypes was evaluated with 19 ISSR and 12 URP (Universal Rice Primers) markers. 92.73 per cent of the ISSR amplicons were polymorphic and PIC values ranged from 0.23 to 0.47 with an average of 0.34. The resolving power (RP) ranged from 7.00 to 16.00 with average of 11.20. The marker index (MI) ranged from 0.77 to 4.83 with an average of 2.93 (Panwar *et al*., 2018).

In African marigold, polymorphism realized using RAPD markers was higher (80-100 per cent, average 98.80 per cent) compared to *Tagetes minuta* (7.0 per cent), *Tagetes patula* (70 per cent), sunflower (69.51 per cent) and *Chrysanthemum morifolium* (89.7 per cent). Hence it is evident that *Tagetes erecta* species is having more diversity in comparison with other related species (Panwar *et al*., 2017).

Similar study was conducted in *Tagetes erecta* for identifying the SCAR marker linked to the male sterile gene *Tems*. 38 ISSR and 170 SRAP primer combinations were screened on two bulks from F_2 population but no polymorphic bands were found in ISSR maker analysis (He *et al*., 2009). In a similar study, a SCAR marker linked to bacterial wilt in tomato was developed through BSA (Truong *et al*., 2015). DNA from six each of resistant and susceptible F_9 RILs, originated from the cross of *S. lycopersicum* Hawaii 7996 (resistant parent) and *S. pimpinellifolium* WVa 700 (susceptible parent), were pooled to create R pool and S pool, respectively. Two RAPD markers tightly linked to bacterial wilt resistance were identified and one was converted SCAR marker.

Future line of work

The present study has revealed three ISSR markers at 750 bp in ISSR 12, 370 bp in ISSR 16 and 150 bp in ISSR 30 associated with bacterial wilt resistance in marigold. These markers can be potentially used for the future bacterial wilt resistance breeding programmes in marigold. This is the first report on the molecular markers linked with bacterial wilt resistance in marigold.

Summary

5. SUMMARY

The research entitled "Identification of molecular marker linked with bacterial wilt resistance in marigold (*Tagetes erecta* L.)" was carried out at Centre for Plant Biotechnology and Molecular Biology, College of Agriculture, Kerala Agricultural University, during the year 2019 to 2021. The objective of this study was to identify an inter simple sequence repeat (ISSR) marker for resistance to bacterial wilt disease in marigold (*Tagetes erecta* L.). Bacterial wilt resistant variety M_1 and susceptible variety Double Yellow were crossed to develop the F_2 mapping population. Artificial inoculation followed by bulked segregant analysis using ISSR marker system was done in F_2 population.

The salient findings of the study are summarized below:

1. M_1 was found highly resistant and Double Yellow was susceptible to bacterial wilt. Pollination was done by slightly cutting the petals surrounding the floral organs, followed by rubbing anthers of male parent M_1 onto the stigma of the female parent Double Yellow for 3 consecutive days. The female flowers were covered with butter paper bags from the colour break stage till harvest.
2. DNA was isolated from both parents through CTAB method and used in polymorphism check.
3. Under natural conditions, F_1 plants had intermediate resistance, suggesting polygenic resistance. Flowers in F_1 plants were covered with butter paper bags and F_2 seeds were harvested.
4. Prior to the planting of 204 F_2 plants, root clipping and dipping of seedlings in bacterial wilt inoculum, having an OD value of 0.9 at 600 nm, were done for 10 minutes. F_2 plants along with the parents were raised under polyhouse conditions and artificially inoculated with freshly prepared bacterial inoculum, by pouring 25 ml of inoculum solution into each pot immediately after planting.
5. 150 of the 204 F_2 plants have shown wilt symptoms and the infection was confirmed by ooze test. Ten each of the most resistant and susceptible F_2 plants along with the parents were subjected to Bulked Segregant Analysis using ISSR marker system.
6. Good quality DNA ($A_{260/280}$ at 1.8-2.0) from 10 each of F_2 resistant and susceptible plants were bulked separately to get the F_2 R bulk and S bulks,

respectively. BSA was performed using DNAs from resistant parent, susceptible parent, resistant bulk and susceptible bulk.

7. Of the 50 ISSR primers screened initially, 21 were polymorphic and hence were used in BSA. Of these 21 primers, eleven primers have generated 179 polymorphic amplicons.
8. Four primers ISSR 12, ISSR 16, ISSR 30 and UBC 866 have yielded eight markers linked with bacterial wilt resistance. The markers at 750 bp (resistance) in ISSR 12, 370 bp (resistance) in ISSR 16, 150 (resistance) and 800 (susceptibility) bp with ISSR 30 and 400 bp (susceptibility), 350 bp (resistance) with UBC 866 were linked with bacterial wilt resistance in marigold.

The markers identified were reproducible and hence they can be used in marker assisted selection for bacterial wilt resistance in marigold.

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Abstract

IDENTIFICATION OF MOLECULAR MARKER LINKED WITH BACTERIAL WILT RESISTANCE IN MARIGOLD

(*Tagetes erecta* L.)

By

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ABSTRACT OF THE THESIS

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Abstract

The annual flower crop marigold has gained popularity due to its easiness of cultivation and wide adaptability. It is grown for loose flowers for garland making, wreaths and religious offerings and is ideal for garden display. It is a rich source of some value added compounds - essential oils, carotenoid pigments *etc*. Bacterial wilt is a major reason for low productivity, especially in Kerala, causing a yield loss up to 65 - 70 per cent under conducive climatic conditions. Moreover, the pathogen being soil borne, management of this disease is very difficult and development of resistant varieties is the most promising strategy.

Bulk sergeant analysis (BSA) is a quick strategy to find molecular markers associated with a trait. In this, plants from segregating population are grouped according to their phenotypic response to the target trait and the marker pattern will be associated with the expression. Usually, F_2 population has been used for BSA because it provides the best recombination and segregation in the population so that extreme phenotypes can be easily distinguished upon artificial inoculation (Michelmore *et al*., 1991).

The study entitled "Identification of molecular marker linked with bacterial wilt resistance in marigold (*Tagetes erecta* L.)" was carried out at the Centre for Plant Biotechnology and Molecular Biology and Department of Floriculture and Landscape Architecture, College of Agriculture, Thrissur during 2019 to 2021. The objective of this research programme was to identify inter simple sequence repeat (ISSR) marker for resistance to bacterial wilt disease in marigold (*Tagetes erecta* L.).

To develop the mapping population, pollen from the resistant line M_1 was used to pollinate the susceptible cv. Double Yellow. F_1 seeds were collected and the hybrids were found to have moderate resistance. Flowers of F_1 plants were selfed by bagging and the seeds obtained were sown to raise the F_2 population. Two hundred and four F_2 plants, resistant and susceptible parents were artificially screened (using fresh bacterial wilt inoculum having an OD value of 0.9 at 600 nm) for bacterial wilt resistance and the most susceptible and most resistant plants in F_2 were identified. Wilting in plants was checked daily and infection was confirmed by ooze test. DNA

was isolated from the parents and 10 plants each from most susceptible and most resistant F_2 plants and S- and R-bulks were prepared.

Fifty ISSR primers were initially screened and 21 primers yielding amplification were selected for BSA. BSA was carried out using parental DNA and S- and R- DNA bulks. A total of 179 amplicons were produced from 21 primers in ISSR marker analysis. From these 11 primers, yielded 23 polymorphic bands and four molecular markers were able to produce eight polymorphic bands segregating with bacterial wilt resistance.

The markers ISSR 12 (750 bp), ISSR 16 (370 bp), ISSR 30 (150 and 800 bp) and UBC 866 (400 and 350 bp) were found segregating with the expression of resistance. Of these, markers ISSR 12 and ISSR 30 were associated with the susceptibility and the rest were associated with the resistance. Six markers identified in this study through BSA can be used in marker assisted selection for bacterial wilt resistance in marigold.

സംഗ്രഹം

വാർഷിക പുഷ്പ വിളയായ ചെണ്ടുമല്ലി പ്രസിദ്ധി നേടിയത് വളർത്താനുള്ള എളുപ്പം കൊണ്ടും, പരിസ്ഥിതിയോട് ഇണങ്ങാനുള്ള കഴിവു കൊ ുമാണ്. ഇത്തരത്തിൽ ഉൽപാദിപ്പിക്കുന്ന പൂക്കൾ പൂമാലകൾ, റീത്തുകൾ എന്നിവ നിർമ്മിക്കുവാനും, മതപരമായ ആവശ്യങ്ങൾക്കും, പൂന്തോട്ട പ്രദർശനങ്ങൾക്കുമായി ഉപയോഗിച്ച് വരുന്നു. പലതരം മൂല്യവർദ്ധിത വസ്തുക്കളായ സുഗന്ധതൈലങ്ങൾ, കരോട്ടിനോയ്ഡ് സംയുക്തങ്ങൾ എന്നിവയുടെ സമ്പന്നമായ ഉറവിടമാണ് ചെണ്ടുമല്ലികൾ. ബാക്ടീരിയ മൂലം ചെടികളിലുണ്ടാകുന്ന വാട്ടമാണ് പ്രധാനമായും കേരളത്തിൽ ചെണ്ടുമല്ലികളുടെ ഉൽപാദനം കുറയാൻ കാരണം. രോഗം പടരുന്ന അനുകൂല സാഹചര്യങ്ങളിൽ 65-70% വരെ വിളനഷ്ടം സംഭവിക്കാം. കൂടാതെ രോഗകാരി മണ്ണിൽനിന്നും ഉൽഭവിക്കുന്നതിനാൽ രോഗനിയന്ത്രണം ബുദ്ധിമുട്ടേറിയതും പ്രതിരോധശേഷിയുള്ള ഇനങ്ങൾ ഉല്പാദിപ്പിക്കുന്നത് ഏറ്റവും ഫലപ്രദമായ മാർഗ്ഗവുമായി കണക്കാക്കപ്പെടുന്നു.

ചെടിയുടെ സ്വഭാവഗുണങ്ങളുമായി ചേർന്ന് കാണപ്പെടുന്ന മോളിക്കുലാർ മാർക്കറുകൾ വേഗത്തിൽ കണ്ടെത്തുന്നതിനായി ഉപയോഗിക്കുന്ന രീതിയാണ് ബൾക്ക് സെഗ്രിഗൻ്റ് അനാലിസിസ്. ഇതിൽ വ്യത്യസ്ത വിഭാഗത്തിൽപ്പെടുന്ന ചെടികളെ വേണ്ട സ്വഭാവത്തിൻ്റെ ഭൗതികമായ പ്രത്യേകതകളുടെ അടിസ്ഥാനത്തിൽ തരംതിരിക്കുകയും മാർക്കറുകളുടെ ക്രമം ഉദ്ദേശിക്കുന്ന ഗുണത്തിൻ്റെ പ്രകടനത്തിനനുസരിച്ച് വ്യത്യാസപ്പെടുന്നതായും കാണുന്നു. സാധാരണയായി എഫ് 2 വിഭാഗത്തിൽപ്പെടുന്ന ചെടികളാണ് ബി.എസ്.എ. യ്ക്ക് വേണ്ടി ഉപയോഗിക്കുന്നത്. അത് വഴി മികച്ച പുനസംയോജനവും വേർപെടുത്തലും സാധിക്കുകയും അങ്ങേയറ്റം വ്യത്യാസപ്പെട്ട ചെടികളെ വളരെ എളുപ്പത്തിൽ വേർതിരിക്കാനും കഴിയുന്നു.

ചെണ്ടുമല്ലിയിൽ കാണുന്ന ബാക്ടീരിയൽ വാട്ടം എന്ന രോഗത്തിനുള്ള പ്രതിരോധ ശേഷിയുമായി ബന്ധപ്പെട്ട മോളിക്കുലാർ മാർക്കറുകൾ കണ്ടെത്താനുള്ള പഠനം നടന്നത് സെൻ്റർ ഫോർ പ്ലാൻ്റ് ബയോടെക്നോളജി ആൻഡ് മോളിക്കുലാർ ബയോളജി വിഭാഗത്തിലും, ഡിപ്പാർട്ട്മെൻ്റ് ഓഫ് ഫ്ലോറികൾച്ചർ ആൻഡ് ലാൻഡ് സ്കേപ്പ് ആർക്കിടെക്ച്ചർ വിഭാഗത്തിലുമായി 2019-2021 കാലഘട്ടത്തിലാണ്. ചെണ്ടുമല്ലിയിൽ ബാക്ടീരിയ മൂലമുണ്ടാകുന്ന വാട്ട രോഗവുമായി ബന്ധപ്പെട്ടു കാണുന്ന ഇൻറർ സിംപിൾ സീക്വൻസ് റിപ്പീറ്റ് മാർക്കറുകൾ കണ്ടെത്തുന്നതായിരുന്നു പഠനോദ്ദേശം.

പഠനത്തിനായുള്ള ചെടികൾ ഉണ്ടാക്കുന്നതിനായി പ്രതിരോധ ശേഷിയുള്ള ഇനമായ എം-1 ൽ നിന്ന് പൂമ്പൊടി പ്രതിരോധശേഷി ഇല്ലാത്ത ഇനമായ ഡബിൾ യല്ലോയിലേക്ക് പരാഗണം ചെയ്യുകയും ഇത്തരത്തിൽ ഉണ്ടാകുന്ന ചെടികളിൽ നിന്നും എഫ് 1 വിത്തുകൾ ശേഖരിക്കുകയും ചെയ്തു. ഇത്തരത്തിൽ ഉൽപാദിപ്പിച്ച ചെടികൾ ഭാഗികമായി പ്രതിരോധശേഷി കാണിക്കുകയുണ്ടായി. എഫ് വൺ ചെടികളിലെ പൂക്കളെ മൂടിവെയ്ച്ച് സ്വയം പരാഗണത്തിന് അനുവദിക്കുകയും, ഇത്തരത്തിൽ ലഭിച്ച വിത്തുകൾ എഫ് 2 വിഭാഗം ചെടികൾ ഉണ്ടാകുവാനായി ഉപയോഗിക്കുകയും ചെയ്തു.

ഇത്തരത്തിൽ ലഭിച്ച 204 ചെടികളേയും ബാക്ടീരിയ വാട്ടം ഉണ്ടാക്കുന്ന രോഗാണു കൃത്രിമമായി നൽകി അവയിൽനിന്ന് അങ്ങേയറ്റം പ്രതിരോധശേഷി കാണിക്കുന്നവയെയും പ്രതിരോധശേഷി തീരെ കുറഞ്ഞ ഇനങ്ങളെയും വേർതിരിച്ചെടുക്കുകയും ചെയ്തു. ചെടികളിലെ ഇത്തരത്തിലുള്ള പ്രത്യേക വാട്ടം സ്ഥിതീകരിച്ചത് ഊസ് ടെസ്റ്റ് ഉപയോഗിച്ചാണ്. പ്രജനനത്തിനു പയോഗിച്ച ഇനങ്ങളിൽ നിന്നും, എഫ് 2 വിഭാഗത്തിലെ ഏറ്റവും കൂടുതൽ പ്രതിരോധശേഷി ഉള്ളതും ഏറ്റവും കുറവ് പ്രതിരോധശേഷി ഉള്ളതുമായ ഇനങ്ങളിൽ നിന്നും 10 വീതം ചെടികളിൽ നിന്നും ഡി.എൻ.എ. കൾ വേർ തിരിച്ചെടുക്കുകയും അതുപയോഗിച്ച് എസ് ബൾക്കും ആർ ബൾക്കും ഉണ്ടാക്കുകയും ചെയ്തു.

തുടക്കത്തിൽ 30 പ്രൈമറുകൾ സ്ക്രീൻ ചെയ്യുകയും അതിൽ നിന്നും ആംപ്ലിഫിക്കേഷൻ കാണിച്ച 21 പ്രൈമറുകൾ ബി.എസ്.എ. യ്ക്കായി ഉപയോഗപ്പെടുത്തുകയും ചെയ്തു. പ്രജനനത്തിനായി ഉപയോഗിച്ച ഇനങ്ങളുടെ ഡി.എൻ.എ. യും, എസ് ബൾക്ക്, ആർ ബൾക്ക് ഡി.എൻ.എ. കളും ഉപയോഗിച്ച് ബി.എസ്.എ. നടത്തുകയുണ്ടായി. ഇത്തരത്തിൽ 21 പ്രൈമറുകൾ ഉപയോഗിച്ച് നടത്തിയ പരീക്ഷണത്തിൽ 179 ആംപ്ലിക്കോണുകൾ ലഭിക്കുകയുണ്ടായി. ഇതിൽ നിന്നും 11 പ്രൈമറുകൾ 23 വ്യത്യസ്തമാർന്ന ബാൻഡുകൾ നല്കുകയും അതിലെ നാല് പ്രൈമറുകൾ ബാക്ടീരിയൽ വാട്ടത്തെ ചെറുക്കുന്ന ജീനുകളുമായി ബന്ധപ്പെട്ട് കാണപ്പെടുന്നുവെന്ന കണ്ടെത്തുകയും ചെയ്തു.

ഐ.എസ്.എസ്.ആർ.12 (750, 500, 480 ബി.പി.), ഐ.എസ്.എസ്.ആർ.16 (370 ബി.പി.), ഐ.എസ്.എസ്.ആർ.30 (150,800 ബി.പി.), യു.ബി.സി.866 (400, 350 ബി.പി.) എന്നീ മാർക്കറുകളാണ് പ്രതിരോധശേഷിയുമായി ബന്ധപ്പെട്ട കാണപ്പെടുന്നത്. ഇതിൽ ഐ.എസ്.എസ്.ആർ.12, ഐ.എസ്.എസ്.ആർ.30 എന്നിവ പ്രതിരോധശേഷി കുറഞ്ഞ ചെടിയിലെ ജീനുമായും മറ്റുള്ളവ പ്രതിരോധശേഷി

കൂടിയ ചെടികളിലെ ജീനുമായും ചേർന്ന് കാണുന്നു എന്ന് കണ്ടെത്താൻ കഴിഞ്ഞു. ഈ പഠനത്തിൽ നിന്നും ചെണ്ടുമല്ലിയിലെ ബാക്ടീരിയ മൂലമുണ്ടാകുന്ന വാട്ടത്തിനുള്ള പ്രതിരോധ ശേഷിയുമായി ബന്ധപ്പെട്ട 8 മാർക്കറുകൾ കണ്ടെത്താൻ കഴിഞ്ഞു.