UTILIZATION OF SYNTHETIC TETRAPLOIDS

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Abstract: Groundnut (*Arachis hypogaea* L.) has a narrow genetic base due to the series of bottlenecks during the evolution of the crop. As a result the crop is susceptible to a range of foliar diseases and insect pests. Levels of resistance to many of the biotic constraints are not up to the desired level in the cultivated germplasm. ICRISAT has taken lead to develop a range of tetraploid (amphidiploid and autotetraploid) also called synthetic groundnut by combining A and B genome of *Arachis* species. Many of these tetraploids are being used to improve groundnut. One such synthetic tetraploid was utilized to develop advance back cross population. The cultivated teraploid (ICGV87846) was crossed with synthetic tetraploid ISATGR 278-18. ISATGR 278-18 comprises of two *Arachis* species namely *A. duranensis* and *A. batizocoi*. BC_2F_2 progeny obtained from the above cross were selfed and were evaluated for a few diseases namely late leaf spot and rust and were also screened for *Spodoptera litura* resistance.

Keywords: Cultivated groundnut, Synthetic tetraploid groundnut, Wild *Arachis* species, Wide crosses, inter specific hydridization.

Introduction: Cultivated groundnut/ peanut (*Arachis hypogaea*) is an allotetraploid (2n= 4x = 40) widely grown in more than 100 countries in both tropical and sub tropical regions of the world. Globally it is cultivated on nearly 24 million hectares of the land area with an annual production of 38 million tons [1]. China leads in production of groundnut followed by India, United States and Nigeria [2]. These seeds are important for oil and play a major role in malnutrition as it provides good source of protein, nutrition, vitamins (thiamine, riboflavin and niacin) [3]. In addition, this crop provides an important live stock feed.

The narrow genetic base in cultivated groundnut is due to the single hybridization event that occurred 3500 years back [4]. Many biotic, abiotic stresses along with narrow genetic base seriously hampered the possibility of meeting future demands of continuously increasing humans and animal population. The two fungal diseases namely Late leaf spot (LLS) caused by *Cercosporidium personata* [(Berk. and Curt.) Deighton] and rust caused by *Puccinia arachidis* , among biotic stress are widespread. These diseases often occur together and cause severe damage to the yield (50- 70%) and also adversely affect the quality of the pod and haulm [5]. Urgent attention is required to reduce these foliar diseases. Commercially many fungicides are available to control these diseases, but it is not a viable option for poor farmers and also usage of fungicides will pollute the ground water and environment which in turn cause greater risk and damage to the crop [6]. So development of host – controlled resistance is considered the best method.

The large range variability is observed in 80 different species placed in nine sections in genus *Arachis* [7] but this variability cannot be exploited directly, because of ploidy difference. To overcome the draw backs of the narrow genetic base, scientists were anxious to develop more tetraploid groundnut by imitating what happened in nature and produce new tetraploids by producing more diploid hybrids using the different combinations of *Arachis* wild species. This was the genesis for the development of synthetic or new source of tetraploid groundnut. For the successful utilization of wild *Arachis* species in the introgression of useful/resistance genes into the cultivated groundnut, few approaches are available [8] to overcome the genomic imbalances with different ploidy levels in the species. Many superior varieties resistant to diseases were developed my groundnut breeders globally, such as NemaTAM and Coan [9] resistant to root-knot are released; GPDB4 is an important variety with desirable traits such as foliar disease resistant and high yield. The present study was undertaken to overcome the genetic bottle neck of restricted gene flow and introgression of resistant gene from developed synthetic amphidiploids ISATGR 278-18 to cultivated groundnut ICGV 87846 by back crossing and self pollination. Identification of resistant and susceptible, in introgression lines is based on the Co-occurrence, defoliation, Partial and polygenic nature of LLS and susceptible lines through conventional screening technique [10]. Screening of *Spodoptera litura* resistance was also done in this population.

Materials and Method:

- ICGV 87846: It is an important Virginia branch type with yellow color flower, double seeded with prominent reticulation. It was derived from a cross of CS9 (ICGV88241 x ICGS (ICGV87121). It is medium duration type, maturing in 125-130 days and high yielding. But it is susceptible to foliar diseases.

- ISATGR278-18 is one of the new sources of synthetic tetraploid developed in ICRISAT Hyderabad by Nalini Mallikarjuna 2011[11]. It was derived from cross of *A. duranensis* (ICG 8138) × *A. batizocoi* (ICG 13160).

Seeds of amphiploid ISATGR 278-18 and cultivated groundnut ICGV 87846 were planted in a glasshouse. The cultivated groundnut ICGV 87846 was used as female plant and ISATGR 278-18 as male plant. Emasculation of ICGV 87846 was done a day before pollination, cross pollination was carried out before 10:00am on the next day using synthetic tetraploid ISATGR 278-18 pollen. Gibberilic acid (GA_3) (0.5ml; 75mg/L) impregnated cotton swab was wrapped around the base of the pollinated pistils. Hybrid pods were harvested and used in the backcross program using ICGV 87846 as the recurrent parent.

BC_2F_2 plants were screened for LLS and rust by the infector row technique in ICRISAT Hyderabad in kharif season (Fig 1). In this technique the seeds were sown in two replicated trials. In each test-plot four rows of test material and one row of ICGV 87846 were sown. ICGV 87846 was used a susceptible check to late leaf spot and rust, and used as infector row. The uridinospores released from infector row spread throughout the field. Fields were irrigated whenever required until harvesting. The LLS and Rust scores were taken on 90th day with a modified 9 point field scale [12] .According to the modified 1-9 scale, 1 (0) indicated no disease, 2 was with(1-5 %) pustules sparsely distributed, largely on lower leaves, 3 was (6-10%) with many pustules on lower leaves, with evident necrosis, and with very few pustules on middle leaves, 4 (11-20%) was with pustules on lower and middle leaves, severe necrosis on lower leaves, 5 (21-30%) was severe necrosis of lower and middle leaves, with the possibility of pustules on the top leaves but less severe, 6 was extensive damage (31-40%) to lower leaves, middle leaves, necrosis accompanied by dense distribution of pustules, pustules on top leaves, 7 (41-60%) showed severe damage of the lower and middle leaves, with pustules densely distributed on top leaves, 8 (61-80%) was100 per cent damage to lower and middle leaves, pustules on top leaves, which were severe, in 9 (81-100%) almost all leaves withered, bare stems were seen.

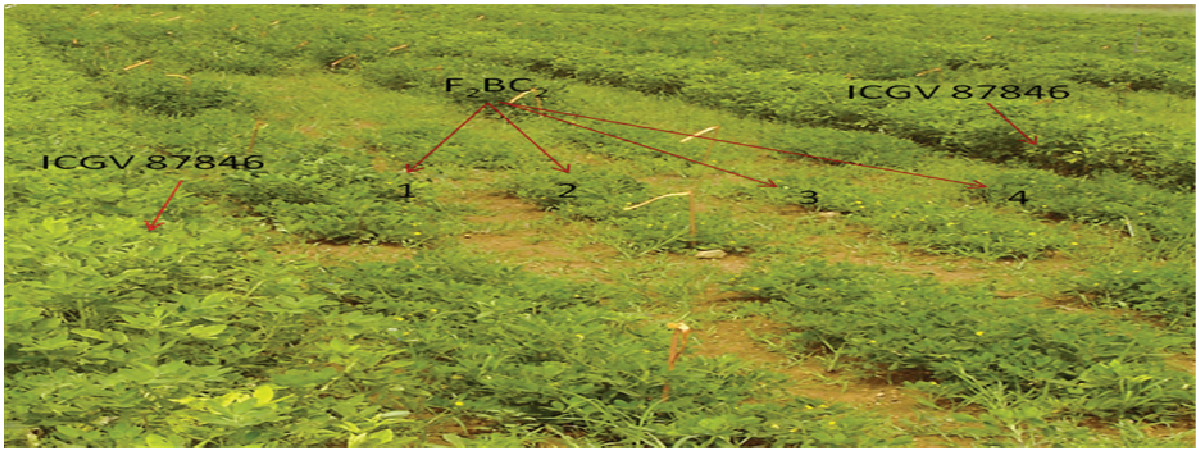

Fig1: Advance screening by Infector row technique

Assay for Proteinase Inhibitors:

Proteinases and Substrates Extraction of crude seed protein:

The plants with highest number of pods from 32 lines were selected for protein estimation and proteinase inhibitors. Mature seeds of BC_2F_2 were collected decorticated and ground to a fine powder/paste in a mortar and pestle. This fine powder was defatted by 2 washes with acetone and 3 washes with hexane. Then the solvent was filtered off. 1% polyvinyl pyrolidine (PVP) was then added in the ratio of 1:6-wt /v and kept for mild continuous stirring for overnight at 4^{o}C. Suspension was centrifuged twice at 10000 rpm for 20 mins at 4^{o}C. Supernatant was collected in aliquote and frozen at -20^{o}C for the analysis of inhibitors of trypsin and chymotrypsin.

Protein estimation: Protein content was determined by Folins-Ciocalteau method using BSA as a standard. The working solution consist of 4% Na2CO3 in 0.2 N NaOH: 2% Sodium potassium tartarate: 1% CuS04 which were mixed freshly (23:1:1) before adding to the sample to be tested.

Assay for Proteinase Inhibitors: Proteinases and Substrates Bovine pancreatic trypsin and bovine pancreatic a chymotrypsin was secure from Sisco Research Laboratory, Mumbai, India .Where as BAPNA (*N*-a-Benzoyl-dl-arginine-*p*-nitroa- nilide)and n- GLUPHEPA (glutaryl-l-phenylalanine-*p*-nitroa-nilide) from Sigma. Trypsin or chymotrypsin inhibitor activity was determined by using appropriate volumes of crude protein extract which

resulted in 40% - 60% decrease in corresponding enzyme activity. Assay mixture (1.0 ml) consisted of protein inhibition sample and assay buffer, (50 mM Tris- HCL containing 20 mM CaCl2) the pH was adjusted to 8.2for trypsin, where as 7.8 pH for chymotrypsin. 10 µg of trypsin or 70 µg of chymotrypsin was added to the assay mixture and incubated for 15 min at 37°C. Residual proteinase activity in the above assay mixture was determined after incubating for 45 min at 37°C using 1 mM BAPNA (1.0 ml) as a substrate for trypsin and 1 mM GLUPHEPA (1.0 ml) as a substrate for chymotrypsin . The reaction was terminated by adding 0.2 ml of 30% acetic acid. The activity of proteinase inhibitors was expressed as trypsin inhibitors (TI) units/mg protein or chymotrypsin inhibitor (CI) units/mg protein. One TI or CI unit was defined as the amount of inhibitor required to inhibit 50% of the corresponding enzyme activity. BAPNA and GLUPHEPA were dissolved in dimethyl sulphoxide.

Extraction of Gut proteinases: The 4th and 5th day larvae reared on the host and non host plants were taken into lab. Larvae were starved for 5hrs and killed by decapitation to collect midgut. Extract the midgut content in 0.2 M glycine NaOH buffer p^H 10.0. The extraction was allowed to stand for 15 min at 4°C. Then the suspension was centrifuged at 12000rpm for 15 min.2 times at 4°c. Supernatant was frozen in aliquots and used for analysis of proteins and proteinases.

Results: Development of Advance back cross population (Fig 2)

When cultivated groundnut ICGV 87846 was crossed with synthetic ISATGR 278-18, 7 F_1 pods were obtained. All F_1 seeds obtained from F_1 plants were planted. The true hybrids were selected based on the morphological traits. Since the parental genotypes were diverse in several morphological traits, identification of hybrid pods were easy as they show intermediate morphological characters of the parents such as growth habit, leaf color, flower color and pod shape and involved in first back cross. After verification 2 plants were true F_1 hybrids. A total of 70 pollinations were done in two F_1 plants from which 13 pods were obtained and planted in the glass house. Out of 13 plants 7 plants were selected which are true BC_1F_1 hybrids and involved in second back cross. Of the 826 buds pollinated in 7 BC_1F_1 plants, 116 crossed pods were harvested and 97 seeds were obtained. These all 97 seeds were planted and subjected to phenotypic screening and identified 33 true backcrossed BC_2F_1plants and subjected to selfing in the next generation. The phenotypic screening was done to this advance back crossed population and more variations were observed (Fig 3).

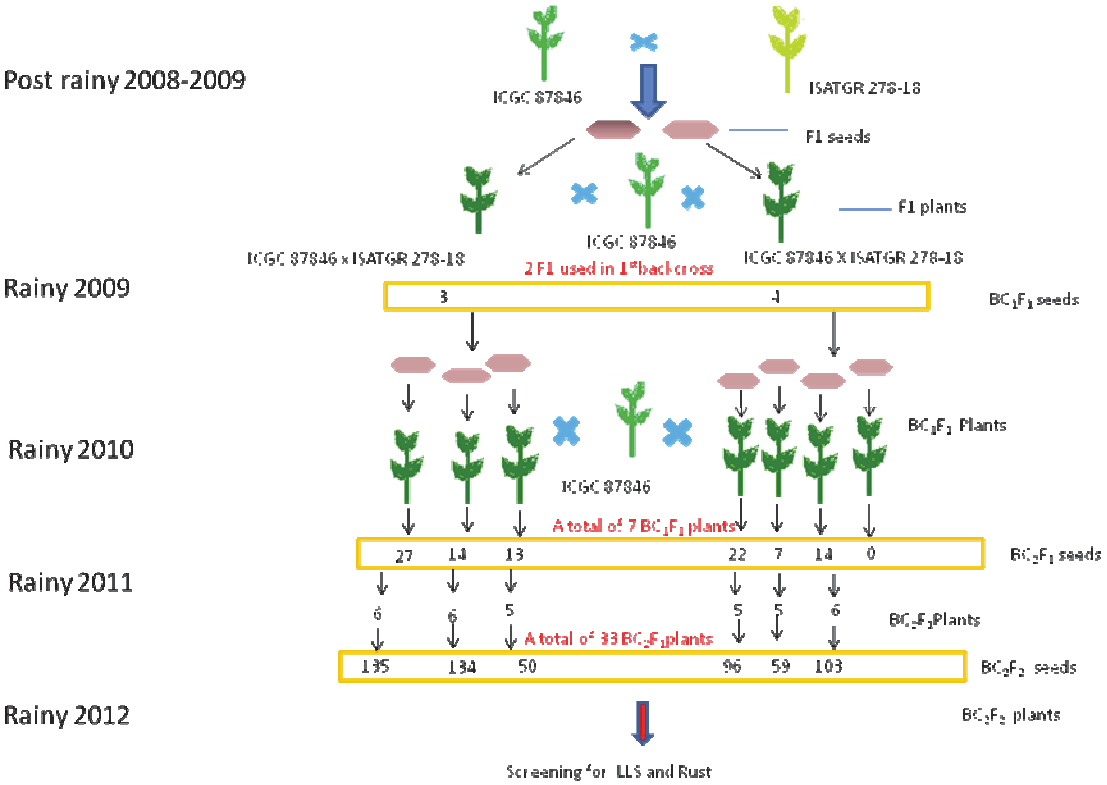

Fig 2: Development of Advance back cross population.

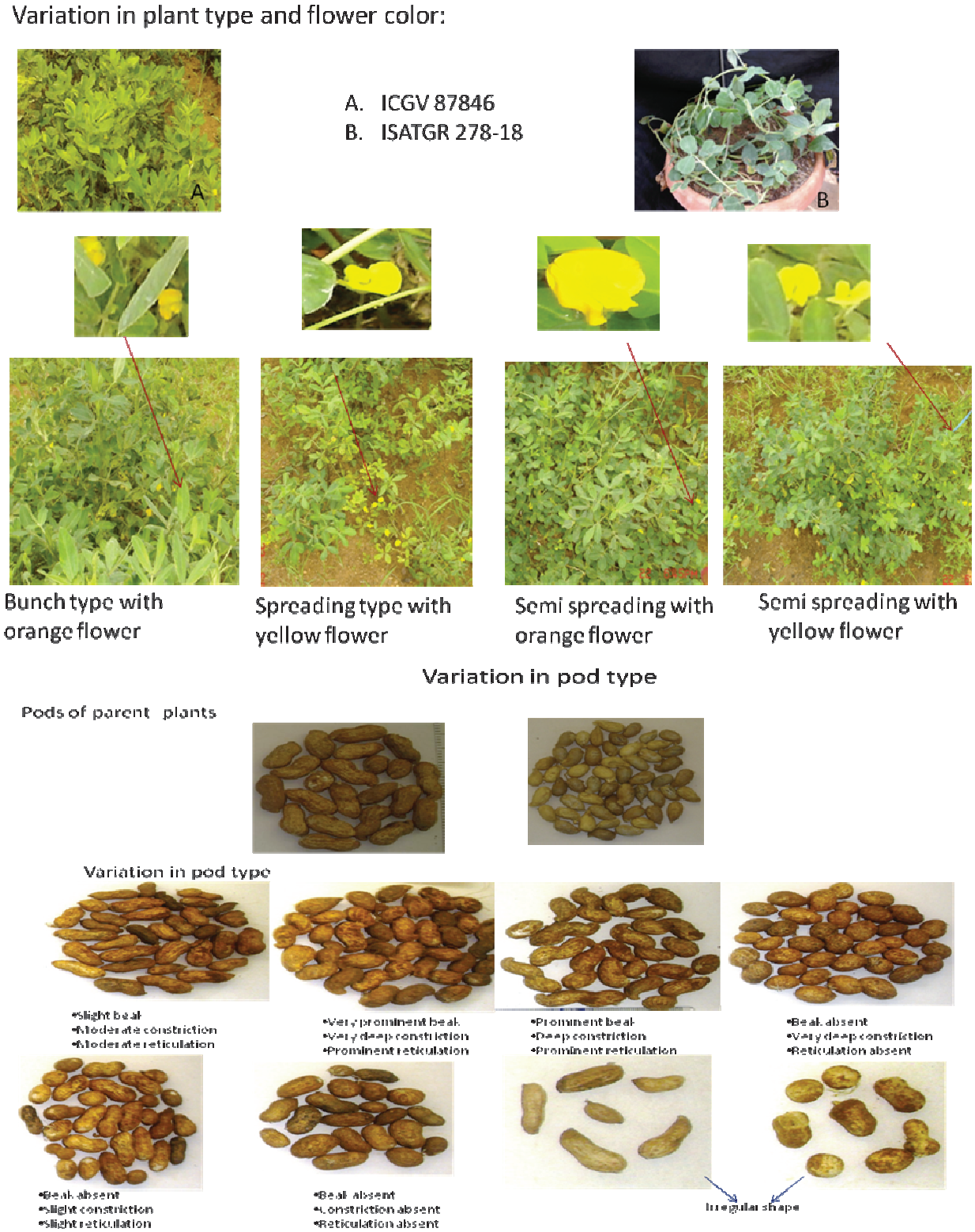

Fig 3: Morphological variations observed in Advanced back crossed population

The BC_2F_2 seeds were sown in 32 lines in two replications for LLS and Rust screening (Fig 4). Out of 32 lines 20 lines in replication I had a score of 3 and remaining 12 lines had a score of 4. In replication II, 2 lines had a score of 2 and remaining 24 lines scored 3 and 7 lines scored 4 for late leaf spot in 9- point scale. In rust screening in replication one, 1 line scored 3, remaining 31 lines scored 2 and in replication II, all 32

lines scored 2.

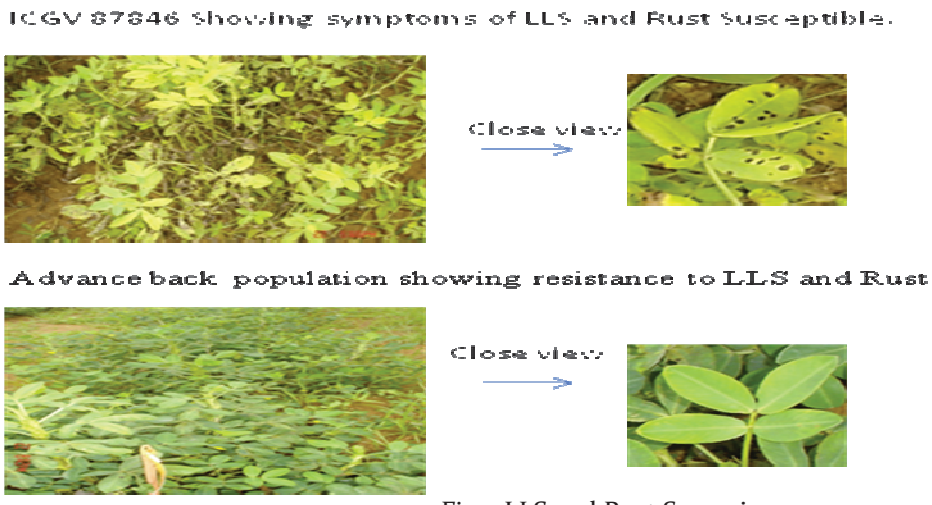

Fig 4 LLS and Rust Screening

Protein estimation and protienase inhibition assay: The cultivated groundnut showed 153 mg/gm protein content, 2.28 T1 units, 2.12 Ci units and 1.34 gut units and in ISATGR 278-18 it was 120.5mg/gm protein content, 2.78 Ti units, 2.32 Ci units and 1.34 gut units.

In advance back cross population the protein estimation and protienase inhibition assay results indicated that, 17 out of 32 samples showed more protein content than the parent's involved in the development of BC_2F_2 population. Maximum protein content was observed in 354 sample number with 355 mg/gm. In Trypsin inhibition assay 23 samples of the population observed more TI units than the parents, a maximum unit was recorded in sample number 392-6 with 8.15 Ti units. Whereas in Chymotrypsin inhibition assay 10 samples showed more Ci units compared to parents, 2.56 Ci units was the highest unit recorded by sample number 388. In gut protiensae inhibition assay 21 samples from 32 showed more gut units than the parents, 10 samples showed equal reading to that of parents. Sample number 412 recorded highest reading of 2.56 gut units.

Discussion: Cultivated groundnut is susceptible to late leaf spot (LLS) caused by *Phaeoisariopsis personata* [(Berk. & M. A. Curtis) Aex]; and the resistance is low to moderate in the primary gene pool of groundnut [13]. Closely related wild species in the secondary gene pool are highly resistant to the disease [14]. Limited success was observed when wild *Arachis* species were used directly for improving the cultivated germplasm. So development of synthetics by Nalini Mallikarjuna in ICRISAT is an effective way for introgression of useful traits from *Arachis* species into the cultigens and is a straight forward process, Based on this BC_2F_2-population was developed from cultivated groundnut ICGV 87846 and synthetic ISATGR 278-18. High level of morphological variations were observed in synthetic derivatives due to abnormal pairing during meiotic division in synthetic ISATGR 278-18 [15] resulted in different types of allelic combinations in the segregating backcross population. When this population is subjected to screening for late leaf spot and rust by infector row method, the uridinospores released by infector row (ICGV87846) easily spreads through wind, irrigation, insects etc to the test plants. The longer incubation period, fewer lesions and lower sporulation of pathogen and less defoliation on the host are the reason that gives resistance for late leaf spot [16]. The score of modified 9-point scale of BC_2F_2 population recorded as 2 in rust indicates that, lesions are present largely on lower leaves and no defoliation and 2-3 in late leaf spot indicates that lesions are present largely on lower leaves, very few on middle leaves and defoliation of some leaflets occurs on lower leaves. From the modified 9-point scale it is clear that this population is resistance to both rust and late leaf spot.

Presence of protein inhibitor of trypsin in plant material was first identified by [17]. They first identified in soya bean, the ability of trypsin to liquify the gelatin was inhibited by aqueous extract of soya bean flour. [18], [19] partially purified the protein with this effect from soya bean [20] isolated this in crystalline form. Protein inhibitors (P Is) prevent the target insect from digesting protein by competitively binding to the active site of protein, which is the actual binding site of proteinase. As the insect cannot digest protein, it is subjected to starvation or even death [21].The protein inhibitors also cause increased levels of insect deformity, due to the potential inhibition of the proteinases involved in

metamorphosis of the larvae [22]. The presence of P I in the synthesized tetraploids, and BC_2F_2 showed that they may play a major role in pest resistance.

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