ANATOMICAL AND GENETIC ANALYSIS OF DAMAGED GRAINS IN RICE (*Oryza sativa* L.)

Thesis

Submitted to the Punjab Agricultural University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE
in
PLANT BREEDING AND GENETICS
(Minor Subject: Biotechnology)

By

Ritika Batra
(L-2016-A-114-M)

Department of Plant Breeding and Genetics
College of Agriculture
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CERTIFICATE I

This is to certify that the thesis entitled **“Anatomical and genetic analysis of damaged grains in rice (*Oryza sativa* L.)”** submitted for the degree of **M.Sc.** in the subject of **Plant Breeding and Genetics** (Minor subject: **Biotechnology**) of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Ritika Batra** (L-2016-A-114-M) under my supervision and that no part of this thesis has been submitted for any other degree.

The assistance and help received during the course of investigation have been fully acknowledged.

(Dr. Gurjit Singh Mangat)
Major Advisor
Senior Rice Breeder-cum-Head
Department of Plant Breeding and Genetics
Punjab Agricultural University
Ludhiana- 141004.

CERTIFICATE II

This is to certify that the thesis entitled **“Anatomical and genetic analysis of damaged grains in rice (*Oryza sativa* L.)”** submitted by **Ritika Batra** (L-2016-A-114-M) to the Punjab Agricultural University, Ludhiana, in the partial fulfillment of the requirements for the degree of **M.Sc.** in the subject of **Plant Breeding and Genetics** (Minor subject: **Biotechnology**) has been approved by the Student’s Advisory Committee along with the Head of the Department after an oral examination on the same.

(Dr. Gurjit Singh Mangat)
Major Advisor

(Dr. Naveen Singh)
External Examiner
Principal Scientist
Division of Genetics
IARI, New Delhi-12

(Dr. Gurjit Singh Mangat)
Head of the Department

(Dr. Gurinder Kaur Sangha)
Dean Postgraduate Studies

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(Ritika Batra)

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Name and Designation of Major Advisor : Dr. Gurjit Singh Mangat
Senior Rice Breeder-cum-Head

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Name of University : Punjab Agricultural University, Ludhiana-141004
Punjab, India

ABSTRACT

Rice grain discoloration or rice black pinhead damage reduces the yield of milled rice and directly reflects on consumer acceptability. The black pinhead damage in grains of PR125 and F_3 population of cross PR121 X PR125 and PR121 X PR126 were studied for its percentage damage intensity, anatomical evaluation and marker analysis. The range of percentage damage intensity for both the crosses was 0.5-7.2 and 0.5- 4.9 per cent respectively. The anatomical evaluation was done using scanning electron microscopy (SEM) and the results showed that there was malformation of starch granules at cellular level. The starch granules of damaged grains were loosely packed while they were densely packed in undamaged ones. 200 SSR markers were applied to check parental polymorphism and 15% (30 out of 200) polymorphism was recorded between parents. Two bulks were prepared for bulked segregant analysis, in which one was with maximum percentage damage and other with no damage. From the above 29 polymorphic markers, only 10% polymorphism was observed in the bulks. Chi square analysis was done to check the inheritance of black pin head damage. Since the statistical analysis followed normal distribution, the damage was attributed to be controlled by two or more genes.

Keywords: Black pinhead, Scanning electron microscopy, Bulked segregant analysis, chi square analysis, starch

Signature of Major Advisor **Signature of the student**

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ਝੋਨੇ ਦੇ ਬਦਰੰਗ ਦਾਣੇ ਜਾਂ ਸਿਰੇ ਤੋਂ ਕਾਲੇ ਹੋਣ ਵਾਲੇ ਚੌਲਾਂ ਦਾ ਝਾੜ ਘੱਟਦਾ ਹੈ ਅਤੇ ਇਸਦਾ ਸਿੱਧਾ ਅਸਰ ਖਪਤਕਾਰਾਂ ਵਿੱਚ ਇਸਨੂੰ ਸਵੀਕਾਰਨ ਉੱਪਰ ਪੈਂਦਾ ਹੈ। PR125 ਅਤੇ PR121 x PR125 ਅਤੇ PR121 x PR126 ਦੇ ਕਰਾਸ ਦੇ F_3 ਜਨਸੰਖਿਆ ਵਿੱਚ ਸਿਰੇ ਤੋਂ ਕਾਲੇ ਦਾਣਿਆਂ ਨੂੰ ਨੁਕਸਾਨ ਦੀ ਪ੍ਰਤੀਸ਼ਤ, ਐਨਾਟੋਮੀਕਲ ਨਿਰੀਖਣ ਅਤੇ ਮਾਰਕਰ ਵਿਸ਼ਲੇਸ਼ਣ ਲਈ ਅਧਿਐਨ ਕੀਤਾ ਗਿਆ। ਦੋਨੋਂ ਕਰਾਸ ਲਈ ਨੁਕਸਾਨ ਦੀ ਪ੍ਰਤੀਸ਼ਤਤਾ 0.5-7.2 ਅਤੇ 0.5-4.9 ਸੀ। ਐਨਾਟੋਮੀਕਲ ਵਿਸ਼ਲੇਸ਼ਣ SEM ਨਾਲ ਕੀਤਾ ਗਿਆ ਅਤੇ ਨਤੀਜਾ ਦਰਸਾਉਂਦਾ ਹੈ ਕਿ ਕੋਸ਼ਿਕਾ ਪੱਧਰ ਤੇ ਸਟਾਰਚ ਵਿਚ ਵਿਸਾੜ ਪਿਆ ਸੀ। ਨੁਕਸਾਨੇ ਗਏ ਦਾਣਿਆਂ ਦੇ ਸਟਾਰਚ ਢਿੱਲੇ ਸਨ ਜਦਕਿ ਸਹੀ ਦਾਣਿਆਂ ਵਿੱਚ ਸਟਾਰਚ ਚੰਗੀ ਤਰ੍ਹਾਂ ਪੈਕ ਸੀ। ਜੰਮਹੇਤੂ ਪੋਲੀਮਾਰਫਿਜ਼ਮ ਨੂੰ ਚੈਕ ਕਰਨ ਲਈ 200 SSR ਮਾਰਕਰ ਦੀ ਵਰਤੋਂ ਕੀਤੀ ਗਈ ਅਤੇ 200 ਵਿਚੋਂ 30 (15%) ਵਿਚ ਪੋਲੀਮੋਰਫਿਜ਼ਮ ਪਾਈ ਗਈ। ਬਲਕ ਸੈਗਰੀਗੇਸ਼ਨ ਵਿਸ਼ਲੇਸ਼ਣ ਲਈ ਦੋ ਬਲਕ ਬਣਾਏ ਗਏ ਜਿੰਨ੍ਹਾਂ ਵਿੱਚੋਂ ਇਕ ਸਭ ਤੋਂ ਵੱਧ ਨੁਕਸਾਨਿਆ ਗਿਆ ਅਤੇ ਇਕ ਬਿਲਕੁਲ ਸਹੀ ਸੀ। ਉਪਰੋਕਤ 29 ਪੌਲੀਮੋਰਫਿਕ ਮਾਰਕਰਾਂ ਵਿਚੋਂ ਸਿਰਫ 10% ਪੌਲੀਮਾਰਫਿਕ ਪਾਏ ਗਏ। ਕਾਈ ਸਕੇਅਰ ਵਿਸ਼ਲੇਸ਼ਣ ਰਾਹੀਂ ਕਾਲੇ ਦਾਣਿਆਂ ਦਾ ਪੀੜੀ-ਦਰ-ਪੀੜੀ ਜਾਣ ਨੂੰ ਦੇਖਿਆ ਗਿਆ। ਸਾਂਖਿਅਕੀ ਵਿਸ਼ਲੇਸ਼ਣ ਨਾਰਮਲ ਡਿਸਟ੍ਰੀਬਿਊਸ਼ਨ ਵਿੱਚ ਸੀ। ਇਸ ਲਈ ਨੁਕਸਾਨ ਨੂੰ ਇੱਕ ਜਾਂ ਇੱਕ ਤੋਂ ਵੱਧ ਜੀਨ ਕੰਟਰੋਲ ਕਰਦੇ ਸਨ।

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ਵਿੱਦਿਆਰਥੀ ਦੇ ਹਸਤਾਖਰ

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CHAPTER- I

INTRODUCTION

Rice (*Oryza sativa* L.) is a cereal crop that belongs to the grass family *poaceae* or *gramineae*. It is a self-pollinating, diploid crop with chromosome number 2n=2x=24. Rice grain quality is the most important factor from marketing and consumer point of view. Rice grains are consumed all over the world and more than 90% Asian population depends upon rice grains for food. Several quality characteristics determine the rice grain quality. Broadly these are hulling and milling recovery (%), endosperm type and chalkiness of endosperm, length and shape of the grain, aroma, amylose content and cooking quality characteristics.

Any damage in rice grains reduces the yield of milled rice and directly reflects on consumer acceptability. The Food Safety and Standard Authority of India (FSSAI) regulations established norms to ensure that such damaged grains (fully damaged/ slightly damaged/ pin point damaged) do not exceed 5 per cent in rice.

Many factors determine the damage in rice grains. As with any cereal grains, rice is subjected to deterioration due to improper storage. The major determinants of storage risk are moisture content, temperature and time of storage (Stockli and Pedersen 1994). Moisture content is said to be the most important factor. In areas where rice is grown, rice yellowing is a serious problem and is a major determinant of quality and price in milled rice (Philips *et al* 1989). Heavy rainfall at the time of grain filling and physiological maturity of rice grains causes discolorations on the rice grains (Reddy *et al* 2004). Rainfall at this time make the crop wet and panicles are more prone to invasion by *Aspergillus* species.

In addition to moisture there are other factors such as temperature, O_2 - CO_2 atmosphere, aeration, pH, and grain condition which interact to affect fungal growth. The loss of nutrients occurs due to changes in lipids, carbohydrates, proteins and vitamins. These factors lead to loss of germination and there are some aesthetic changes like discoloration, caking and abnormal odors (Bothast 1978). A large amount of grain damage is caused during the threshing process in a combine. This process of stripping the grain from the plant causes cracks and other damages. Grain damage is also largely affected by rotor speed.

Rice grain discoloration is another serious threat to rice crop that has great impact on physical properties of rice grains and consumer acceptability (Amini *et al* 2015). Discoloration of rice grains is a major problem in certain cultivars of rice. It reduces the germination and leads to the decay of seedlings, produces chaffy grains and lowers the consumption quality of grains. The disorder may be limited to individual grains, but in severe cases almost the entire panicle including the rachis becomes discolored. It is a complex disease that develops by range of microorganisms including *Drechslera oryzae, Fusarium* sp, *Nigrospora oryzae, Penicillium* sp, *Cuvularia* sp, *Sarocladium oryzae* and species of *Rhizopus* (*http://www.rkmp.co.in/content/grain-discolouration-0*).

Another type of damage due to discoloration is of black pinhead on rice grains. Black pin head, though not common, was earlier observed in widely grown rice cultivar Phalguna variety RP6-17 introduced during 1975 in Andhra Pradesh (Bhat *et al* 1982). In the following years it was observed that about 7% of grains showed black discoloration. The intensity of discoloration was found to be more in parboiled rice (Toteja *et al* 2006*).* The variety was tested for fungal infestation and nutritive value. Both the cases were nullified as it was proved that discoloration was not due to fungal infestation and nutritive value of blackened grains was found similar to normal grains.

A high yielding variety, PAU 201 released by Punjab Agricultural University, Ludhiana in the year 2007, within two years of its introduction, covered approximately 20% area in the state. But this variety was withdrawn from the recommended list of rice varieties for state because of presence of discoloration and black pin point damage on the grains. It was thought that black pin point damage on the milled grains could be due to any fungal contamination. Therefore, stored samples of PAU 201 were milled and analyzed for presence of fungal and aflatoxin contamination. Scanning Electron Microscope (SEM) and Energy dispersive X- ray (EDX) analysis techniques were used to determine fungal spores. Results revealed that black pin point was not the result of any fungal contamination and also not due to aflatoxin (Siruguri *et al* 2012). This character has now been observed in a few advanced and high yielding rice lines of PAU. The character is not found to be localized, but is found at different positions on rice grains. The character is visible only in the milled rice grains and that is why it can affect the acceptability of the cultivar with millers as well as consumers.

Microscopic anatomy involves the use of optical instruments in the study of tissues of various structures known as histology and also in the study of cells. Scanning electron microscopy is one of the tools to study any deformations in the cells. A focused beam of electrons is used to scan the surface of the tissues for imaging. It helps in providing information about the surface topography and sample composition. SEM can examine both hard and dry materials as wood, bone etc. and also the living cells and tissues (Stokes 2008).

Dang and Copeland (2004) also studied that Environmental Scanning Electron Microscopy (SEM) can be used to study the grain structure. SEM allows the grains to be viewed at high resolution with minimum preparation. The milled rice when viewed under SEM showed two distinct features: a smooth surface and a rough surface. The smooth surface had a fracture plane between endosperm cells whereas the rough surface had it passing through the endosperm cells. Parboiled rice showed a glassy fracture surface and features of endosperm were smaller than the uncooked rice. The heated and puffed rice showed complete loss of organizational structure. The study thus concluded that SEM can be used to view various aspects of grain surface.

Though the cause of rice black pinhead is still not clear, the anatomical studies of rice grains with black pinhead may help to identify association of this disorder with any cell developmental stages in the absence of any fungal or bacterial contamination.

Further, molecular analysis of the DNA from damaged and normal grains can reveal some information. Among the various molecular markers available, microsatellite markers also known as SSR markers have expressed as a powerful tool in genotyping. These are simple tandem repeats of di to tetra nucleotide sequence motifs which are flanked by unique sequences. These are basically the non- coding regions and are valuable as genetic markers because of their co-dominant nature. These markers play a vital role in detection of polymorphic loci. Being a model crop plant, rice is rich in genetic and genomic resources that can be used to study genetic architecture of important traits.

Bulked segregant analysis is the study in which DNA from segregating populations of a single cross are pooled or bulked and each individual of the bulk is identical to another one for a specific genomic region or a gene under study (Michelmore *et al* 1991). Bulked segregant analysis technique is used to identify genetic markers associated with a phenotype. This technique involves forming two groups with contrasting phenotypes for a trait of interest. The samples of DNA are bulked by pooling the DNA of all the individuals in each group.

Bulked segregant analysis (BSA) serves as an affordable strategy for mapping large effect QTLs by genotyping only the extreme phenotypes instead of the entire mapping population. It also helps in saving cost by genotyping pooled DNA from groups of individuals with similar phenotypes (Salunkhe *et al* 2011).

The present study has been designed to elucidate the cause and genetic control of black pinpoint on the rice grains complementing electron microscopy analysis of damaged rice grains and BSA to find associated molecular marker.

CHAPTER- II

REVIEW OF LITERATURE

Rice grain is subjected to damage as any other cereal grain. Damage in grains directly affects its milling yield, price, food product quality and consumer acceptability. Grain damage can be defined as any kind of degradation in the grain quality. From the sowing of the crop to its actual end use, grains go through several handling operations which can contribute to grain damage. The losses which occur after harvest are attributed to grain damage only.

Rice grain discoloration and black pinhead are the important damages that occur in rice grains. The presence of black colored spot on the surface of grain can be referred to black pin point damage. On the other hand, rice grain discoloration may occur as a result of fungal infection. Heat damage may also lead to discoloration in rice grains. A noticeable change in color occurs due to heat damage.

In order to understand the reason of damage, techniques like Scanning Electron Microscopy (SEM) and Molecular analysis using molecular markers can be used. SEM analysis determines the surface and internal structural changes that occur inside a grain. SEM can also be used to study the fungal infestation as well as any other kind of microbial contamination. On the other hand, molecular markers can be used to find the association of molecular marker and identification of candidate genes associated with trait of interest. PCR based SSR markers are the most suitable marker system to determine polymorphism between two different genotypes due to their convenient molecular assay and abundance in the genome.

The review of literature has been described under the following headings

2.1: Factors affecting the Rice grain quality

2.2: Rice grain discoloration

2.3: Black pinhead in rice grains

2.4: Bulked segregant analysis

2.1 FACTORS AFFECTING RICE GRAIN QUALITY

There are several different factors that affect rice grain quality. These can be broadly categorized as physiological, biochemical and biological factors, which are discussed as follows:

2.1.1 PHYSIOLOGICAL FACTORS: Factors like temperature, moisture, storage conditions, harvesting techniques, losses during milling etc.

Dillahunty *et al* (2001) studied the effect of temperature, exposure duration and moisture content on color and viscosity of rice. The research was primarily carried on yellowing of Cypress rice. There was great effect of temperature and exposure but effect of moisture content was not significant. Temperature more than 50˚C and exposure duration of more than 12 hours leads to color degradation. The combined effect of temperature and duration was found to be most significant.

Kumar and Kalita (2017) studied the cause and status of post-harvest losses of grains and also discussed the management for these losses. Harvesting was the very first factor. They stated that grain loss at harvesting was very common as most of the harvesting is done manually. The timing and method of harvesting were the two most important factors. In Punjab, wheat harvesting loss due to shattering was 67%. Losses can also occur during threshing and cleaning. These operations, when done manually cause serious damage to grains. Grain loss during winnowing could be as high as 4%. Damage can also occur during drying and storage. Maximum losses were found to happen during storage. Transportation and milling of grains are other causes of grain damage. Milling of paddy resulted in high quantity of brokens and cracked kernels. Milling losses were found to be highest in rice.

Rice grain quality can be assessed based on Physical and milling Characteristics such as moisture content, shape, size, whiteness, translucency, chalkiness, head rice, broken rice, brewers, green kernels and chalky kernels.

Moisture: For storage of time period less than 6 months, the moisture content of 13% is accepted and for long term storage it is 12%. The excessive levels are said to adversely affect the cooking and processing quality characteristics of rice.

Grain Dimensions: International classification (SES, 1996) of rice on the basis of length divides it into 4 classes: Extra-long (> 7.50 mm), long (6.61-7.50 mm), medium (5.51-6.60 mm) and short (≤ 5.50 mm). The grain shape is estimated by length / breadth ratio of kernels and is classified as: Slender (> 3.0), medium (2.1-3.0), bold (1.1-2.0) and round (≤ 1.0).

Grain weight: Relative measure of dockage or presence of foreign material, and the proportion of unfilled, wrinkled and immature kernels is estimated using grain weight.

General appearance: The kernels which are colored, damaged and imperfect ones are not accepted by the consumers. Clear, translucent kernels are highly acceptable by all the segments of rice industry.

Milling yields: The whole grains and grains having ≥ 3/4th of the length are termed as head rice. The rest are said to be broken.

Physico-chemical characteristics: - Amylose content, protein content, gel consistency, water absorption and cooking time.

Organoleptic characters: - Color, aroma, hardness, stickiness and consistency. The scent or natural fragrance in rice grains, particularly Basmati rice is a much valued quality factor. The predominant chemical creating the flavor is 2-acetyl- 1- pyrroline, found in the volatile oil component of the cooked rice.

2.1.2 BIOLOGICAL FACTORS

Sahay and Gangopadhyay (1985) studied the effect of wet harvesting on bio deterioration of rice. They took two varieties of rice- Ratna and Annapurna. These were subjected to four different conditions of processing. The results indicated that the delayed and improper processing of grains along with the occurrence of rains that is in high moisture conditions showed reduction in head rice yield, discoloration of grains and it also lead to fungal infection and release of mycotoxins.

Ashfaq *et al* (2017) studied the grain discoloration disease. They stated that it could be caused by both biotic and abiotic factors such as micro-organisms like fungi, bacteria and viruses and other factors like high moisture content, high temperature, high humidity, immature grain filling, lack of proper pollination and fertilization, chemicals, rainfall at maturity and grain lesions. In Punjab, the disease was studied on Basmati varieties and it appeared on the crop at booting stage at high humidity.

2.2 RICE DISCOLORATION

2.2.1 MICROBIAL CONTAMINATION:

Malaker *et al* (2009) studied the effect of black point disease on quality of rice grains. The study was conducted to analyze the effect of black point infection caused by *Bipolaris sorokiniana* and other pathogens. Grading of wheat grains was done on the scale of 0-5 based on the damage intensity. Grain quality traits like protein, fat, dry matter, ash and mineral contents were determined. The protein and fat contents showed increasing trend with increase in severity whereas the dry matter and ash content showed decreasing trend with increasing severity of black point infection. Macro and micro nutrients were also observed. There was increase in level of N, P, Ca, Mg, S and B and a decrease in levels of K, Fe, Zn, Cu and Na with increasing severity.

Fernandez and Conner (2011) studied various types of kernel discolorations in common and durum wheat. The two main types of discolorations were found to be black point and dark smudge. These were found to be associated with *Alternaria* *alternata* and *Cochliobolus sativus* fungi. The types of discolorations vary significantly based on various environmental conditions during kernel maturation. In most of the cases this trait has been found to be associated with fungal pathogens. The damage was seen primarily at pericarp and testa at the embryo end of the seed.

Williamson (1997) suggested that discoloration in common wheat was not associated with infection caused by fungus *A.alternata*. This study showed that the black point symptom was due to an enzymatic browning caused by phenolic compounds within the grains which are oxidized by peroxidases which further lead to the damage. The resistant and susceptible lines showed the difference in isozymes.

Korndorfer *et al* (2008) discussed the effect of silicon on grain discoloration in upland rice. The experiment was conducted on four different types of soils. The results declared that grain discoloration was reduced with increase in application of silicon. The grain discoloration tends to reduce grain weight, reduce seed germination and lower the grain market value. Several different pathogens were found to be associated with the trait including species like *Curvularia, Helminthosporium* and *Fusarium*.

Jackowiak *et al* (2005) conducted Scanning electron microscope analysis on kernels of five wheat cultivars inoculated with *Fusarium culmorum* conidia. Samples were prepared for SEM analysis and 6 random samples were selected. Surface of kernels showed hyphae in or near the crease and on the side of embryo. Outer layer of kernels had a thick layer of hyphae. Presence of hyphae was also seen in the pericarp and endosperm cavity. Degradation of starch granules was also observed.

Adlakha and Joshi (1974) studied black point of wheat. They defined black point as a black or brown discoloration of the germ end and surrounding areas on the grain. The results declared fungi as the causal organism of the disease in case of wheat but it may also be a result of abiotic stress conditions.

2.2.2 BIOCHEMICAL FACTORS:

Mak *et al* (2006) studied the association of black point of wheat with stress level and defense related proteins. The results declared that abiotic factors caused the black point. Fungal proteins were found to be absent. Decreased levels of stress and disease defense related proteins were observed which suggested that the disease was prevented due to increased levels of stress proteins.

Gras *et al* (1989) suggested that discoloration was mostly influenced by temperature and water activity. They particularly studied the yellowing in rice grains and suggested that yellowing can be achieved artificially. They suggested that non – enzymic browning may lead to yellowing of grains.

Axtell and Baik (2006) studied the effect of phenolic compounds in food product discoloration of barley grains. They determined the phenolic acid and proanthocyanidin content of 11 barley genotypes using Liquid chromatography-mass spectroscopy and High performance liquid chromatography. The grains that were abraded showed higher content of phenolic acid in hulled barley as compared to hull less barley. The discoloration increased on addition of PA extracts to the flour. Catechin content was found to be higher in hull less barley than hulled barley. Catechin showed largest influence on discoloration amongst all other phenolic compounds.

Wang *et al* (2002) took Bengal rough rice to compare the flour and starch structure and properties from yellowed, whole and broken kernels. The discolored or yellowed rice kernels showed higher protein content and low starch yield. Flour prepared from yellowed kernels was studied and showed higher pasting temperature, higher starch damage and reduced amylase content. The results from High performance size exclusion chromatography showed that yellowing could be partially attributed to microbiological activity. It suggested non-enzyamtic browning reaction leading to yellowing of rice. Enzymes degrading the starch and α-amylose played an important role in yellowing by producing reducing sugars that interacted with protein in kernels of rice.

Barthlott (1981) surveyed epidermal surfaces of seed plants. Using SEM, he studied the cellular arrangement or pattern, cell shape, relief of outer cell walls for superficially visible wall inclusions and thickenings and epicuticular secretions mainly waxes and related substances. The arrangement of cells was not clearly visible by SEM; however analysis for cell shape showed various types like isodiametric or elongated cells. Some substances like terpenoids, flavanoids were found to be present as seen after SEM analysis.

Mares and Stone (1973) studied structure and composition of endosperm cell walls. The endosperm cell walls were examined using light, scanning and transmission electron microscopy. For SEM analysis kernel was cut into different cross sections and fixed. Results showed that subaleurone layers of endosperm had smooth walls distorted due to presence of cell contents. Moulding of walls was seen on cells and different patterns of prismatic and central cells were observed. Cell walls possessed microfibril skeleton.

Singotamu and Shetty (1998) observed that rice kernels showed different colours such as chalky, yellow and black with creamy rice. These were believed to have any fungal infection or were due to unseasonal rains, poor storage conditions and high moisture. SEM analysis of these kernels was done using milled rice Hansa. The samples were sorted and were mounted on SEM stub, gold coated in vacuum evaporator and pictures were taken. Pictures revealed that in creamy kernels, starch granules within the endosperm were compactly packed in paracrystalline form. There was no fungal infection. In chalky kernels, starch granules were loosely packed and were granular. Yellow colour was attributed to secondary metabolites released by fungal pathogens.

2.2.3 PHYSICAL OR ENVIRONMENTAL FACTORS:

Sisterna and Sarandon (2000) suggested the high incidence of black point in the conditions of high relative humidity and rainfall. The discoloration ranges from brown to black. Pericarp and testa at the embryo end showed dark brown discoloration. It also occurs along the crease or on the seed coat. Grain quality impairing flour, semolina and other products is greatly reduced (Sisterna and Sarandon, 2010). The incidence of Black point can also be caused by various cultural practices such as nitrogen fertilization (Conner *et al* 1992). Sisterna and Sarandon (1996) suggested that black point incidence can also be attributed to change in tillage systems.

Krishnan and Rao (2005) studied the effect of genotype and environment on seed yield and quality of rice. They took 12 different genotypes of rice viz; IR 36, IR 72, IR 64, Ratna, Swarnaprabha, CR 876-6, CR 691-47, Heera, Radhi, Vanaprava, Annada and Dhola Heera and grown for three yeras. To determine discolored grains they took a subsample of 1000 grains from the three replicates of each sample. The grains were then examined under the magnifying lens. Pink, red, yellow, black colored discolorations were seen. The results determined that the discoloration was affected by environmental factors.

Stockli and Pederson (1994) studied the effect of storage conditions of milled rice on its quality. Two lots of long grain New Bonnet Rice were taken to study the effect of moisture content and length of storage with moisture contents at 14, 18, 22 and 26% . These were stored for 5, 10, 15, 20, 25 and 30 days in an insulated chamber maintained at a temperature of 25˚C and 88% relative humidity. The data obtained suggested that discoloration was high in rough grains stored at high moisture conditions and they had lower milling yield and lower germination percentage. The percent discoloration was determined by dividing kernels by weight of milled rice sample multiplied by 100.

The United States Department of Agriculture described grain damage as degradation in the quality of the grain and classified various types of damage. The damaged grains can be categorized into various categories as broken or cracked kernels, fungal and insect infestation and heat damage, damage during harvest and damage during handling. These damages are caused during grain transport, exposure to fungal spores, drying of grain, using mechanical tools and handling and storage.

Singotamu and Madhusadhanachary (2004) in their study used prosomillets, little millets, barnyard millets and kodo millets for detection of their ultrastructural characteristics and elemental composition by SEM and Energy dispersive X-ray (EDX) method. Ten gram sample of each was taken. SEM studies revealed that all the four millets had paracrystalline form of cells. They stated that SEM and EDX can be used to study both qualitative and quantitative aspects of food grains.

Cytogenetic studies in *Pennisetum* were made by Rau (1929) and Avdulov (1931). For anatomical analysis, they exposed the roots to cyclohexamide 25 mg/l: hydroxyquinoline 300 mg/l (1: 1) for 2 h 45 min and washed the roots in distilled water and fixed in fresh cold ethanol: acetic acid (3: 1) solution. Slides were prepared by using the squashing technique with enzymatic maceration (Pectinase 20%, Cellulase 2%), hydrolysis in HCl 1 N at 60◦C for 10 min, and Fueled staining. High survival of treated explants was observed. These results show that this variation was due to genetic differences between the hybrids genotypes.

Mapiemfu *et al* (2017) studied the quality of rice grains as affected by biophysical and pre-harvest factors. Grain quality attributes such as varietal purity, grains with brown spots, fissured grains, chalky grains were studied as these affected consumer acceptability. The effect of agro-ecological zones was studied. All the characters except brown spot were affected by the agro-ecological zones. The rice in irrigated lowland area had higher weeding frequency than rainfed lowland and upland areas. Brown spot caused by *Bipolaris oryzae* was more prominent in parboiled rice, whereas in non-parboiled samples brown spot severity was less but grains appeared chalky. Fissures were found to be caused due to moisture re-absorption by dried grains.

2.3 BLACK PINHEAD IN RICE GRAINS

Black pinhead or black pinpoint damage is a black dot like structure present on the surface of grain. This discoloration on the rice grain directly affects its milling quality and consumer acceptability. As per FSSAI norms damage intensity should not be more than 5% in rice. Black pinpoint damage reduces the milling yield of the grains and is not accepted by millers. The character was earlier seen in PAU 201 and has now been observed in PR125 as well. The studies conducted regarding the trait have been discussed below.

Siruguri *et al* (2012) conducted a research on rice variety PAU 201 for aflatoxin contamination. The variety was not allowed to be milled or publically distributed in Punjab due to the presence of damaged grains at higher levels than allowed. The permissible limit was 4.75%. The samples were collected from six different districts of Punjab. The samples were milled and analyzed for fungal and aflatoxin contamination using Scanning electron microscopy (SEM), energy dispersive X-ray (EDX) and Prussian blue staining. The aflatoxin analysis concluded that none of the samples exceeded the regulations of Food Safety and Standards (Contaminations, Toxins and Residues). It was confirmed that no fungal structures were present. Presence of iron was also nullified. Only the grains that were fully damaged showed the presence of fungal spores. The study resulted that it did not affect the health of the consumers and it fulfilled the criteria laid down by FSSAI.

Bhat *et al* (1982) studied the nutritional and toxicological aspect of black tip of rice. They conducted the study on Phalguna (RP 6-17) variety which showed black tip symptoms. For the study, milling of brown rice was done and 200 gram of paddy used for parboiling. The results categorized the discoloration to normal, shriveled/discolored and black tip. Samples that were commercially parboiled had the nutrient composition 2-3 times higher in black tipped rice. Unlike this, other parboiled samples did not show much difference in nutritional value. Discoloration intensified on parboiling. Breakage was also higher in the affected grains. Although there was no considerable difference in the nutrient composition of grains but iron and free sugar were found to be higher in the affected grains. Glucosamine content and discoloration were positively correlated which served as a criteria for fungal contamination. *Alternaria* and *Curvularia* were also seen. A study conducted on chicks, ducklings and rats suggested that there were no ill or toxic effects on health of organisms.

2.4 BULKED SEGREGANT ANALYSIS

Bulked segregant analysis is a technique used to identify genetic markers associated with a mutant phenotype. This allows geneticists to discover genes conferring disease resistance or susceptibility. The technique involves the formation of two groups that display opposing phenotype for the trait of interest. The DNA from both the groups is then pooled. The bulked samples thus formed can be analysed using RFLP or RAPD to detect similarities and differences in the various loci of the genome (Henke *et al* 2013). BSA is a rapid and simple method for identifying markers linked to specific genes. It requires the existence of a population which is a result of a cross that segregates for the gene of interest. The success of this technique depends on the genetic divergence between the parents.

Michelmore *et al* (1991) used bulked segregant analysis technique to identify the markers linked to the gene of interest. In the study conducted, two DNA samples obtained from a segregating population of a single cross were bulked. The individuals similar for a given character were pooled together. The screening of bulks was done using restriction fragment length polymorphism probes or random amplified polymorphic DNA primers.

Salunkhe *et al* (2011) studied the fine mapping of QTL for drought resistance in rice. In this experiment, 343 rice microsatellite markers were used to screen the parental lines viz., IR20 and Nootripathu. The markers polymorphic for parents were used to screen resistant and susceptible bulks. The results showed that 96 out of these 343 markers were polymorphic for parents and 7 out of these 96 markers were polymorphic for the bulks.

Govindaraj *et al* (2005) used BSA as a method to detect QTL linked with grain quality traits in Basmati 370 / ASD 16 cross in rice using SSR markers. In this study, a population of 247 F_2 plants was evaluated for their segregation pattern of grain length (GL), grain breadth (GB), cooked grain length (CGL), cooked grain breadth (CGB) and gelatinization temperature (GT). The markers associated with traits from selected bulks were analyzed. The results stated that RM 225 on chromosome 6 and RM 247 on chromosome 12 had association with GB and CGB respectively.

Nematzadeh *et al* (2004) used RAPD markers in BSA for mapping the gene for aroma in rice. F_2/F_3 population of Basmati 370 (aromatic) and IR36 (non-aromatic) was studied. Samples of DNA from homozygous aromatic and non-aromatic plants were bulked and used for BSA. RAPD markers, 550 in number were used to conduct this study. The markers viz., AG8 and AN1 showed polymorphism between aromatic and non-aromatic types. AG8-AR, AN1-AR1 and AN1-AR2 markers were found to be linked with gene responsible for aroma and AG8-AR was found to be located on chromosome 8 of Basmati 370.

Venuprasad *et al* (2009) identified and characterized large-effect QTL for grain yield in conditions of lowland drought stress in rice using bulked segregant analysis. The study aimed at determining if BSA could be used to detect linkage between markers. $F_{4:5}$ population of 490 RIL individuals from cross Apo / Swarna was analyzed to detect the desired QTL using BSA. RM 324 and RM 416 were found to be located on chromosomes 2 and 3 respectively and were tightly linked with yield under lowland conditions. Results stated that BSA could be an effective method for identifying QTL alleles with large effects on yield under stress conditions.

Bulk segregant analysis for improving the drought resistance in maize was done by Quarrie *et al* (1999). Two sets of maize composites (Tuxpeno Sequia, TS and Drought tolerant population, DTP) were selected based on yield under drought conditions for several years. After 8 selection cycles, TS (C8) showed that yield under severe drought was 50% greater than in unselected population (C0). DTP population after 2 selection cycles (C2) was selfed at C5. The top 10% lines were combined to make C6. Bulked segregant analysis was then performed on the 4 populations (TS C0 and C8, DTP C2 and C6). The study suggested that BSA can be used as a tool by plant physiologists in the future.

Subudhi *et al* (1997) used BSA as a technique for molecular mapping of thermosensitive genetic male sterility gene in rice. This was achieved using F_2 population from a cross between a TGMS mutant line (IR32364TGMS) and IR68. Two bulks of fertile and sterile plants were made. RAPD primers, 389 in number were used for BSA. Out of these, four markers were identified to be linked with the trait in question. Three markers namely $OPF18_{2600}$, $OPB19_{750}$ and $OPAA7_{550}$ were identified to be linked to the target gene *tms*3(t) in repulsion phase while $OPAC3_{640}$ was attached to the target gene in coupling phase. $OPF18_{2600}$ and $OPAC3_{640}$ showed polymorphism.

Shen *et al* (2002) detected QTL resistant to Fusarium Head Blight (FHB) in wheat using BSA. Two wheat cultivars viz., Ning 894037 (resistant to FHB) and Alondra (moderately susceptible) were crossed to develop a population of 218 recombinant inbred lines. DNA form 15 most resistant and 15 most susceptible RILs were pooled to create the resistant and susceptible bulks respectively. The two bulks and the parents were screened for polymorphism using 251 SSR markers. Polymorphisms were identified between two bulks and individuals of these bulks were screened for that marker. Ninety nine SSR markers showed polymorphism between parents and out of these 16 markers showed polymorphism between the bulks. The QTL with the large effect was found to be located on short arm of chromosome 3B.

Zhang *et al* (2009) detected QTL related to heat tolerance in rice using bulk segregant analysis. Two rice cultivars were used in this study. The heat tolerant cultivar 996 and the sensitive 4628 were planted. F_2 seeds were obtained. Bulks with extreme phenotypes were made for marker analysis. Each bulk contained DNA from 10 extreme individuals. Polymorphism was checked using SSR markers. 200 SSR primer pairs were used to screen the parents and 61 markers were found to be polymorphic between parents. Two markers RM3735 and RM3586 showed polymorphism between bulks. Single marker analysis showed the presence of these genes on chromosome 3 and 4.

Zhang *et al* (1994) mapped genes for photoperiod sensitive genetic male sterility in rice. The experiment involved a 2- step approach in which firstly chromosome regions containing PSGMS genes were identified from extreme fertile and extreme sterile plants of a very large F2 population. And secondly map locations of these genes were identified. Two chromosomal regions containing PSGMS locus were identified. One was designated as *pms*1on chromosome 7 and the other as *pms*2 on chromosome 3. Marker analysis was then carried out which showed that *pms*1 showed 2-3 times larger effect than *pms*2. It also showed that there was almost complete dominance at both the loci.

Chantret *et al* (2000) detected the location of powdery mildew gene MIRE and a resistance QTL using BSA in wheat. The bulks with extreme phenotypes were constructed from a population of 140 F_3 families obtained by the cross between RE714 (resistant) and Hardi (susceptible). Two bulks each containing 9 individuals showing extreme phenotypes were made for analysis. One hundred and nine microsatellites were tested on RE714, Hardi and 2 bulks. Only 5 primers were found to be polymorphic between the parents and the bulks.

Barua *et al* (1993) used BSA as a tool to identify RAPD markers linked to *Rhynchosporium secalis* (Rh) resistance locus in barley. They used 300 random sequence primers to screen near isogenic lines (NILs) and their parents. One of the primers generated a product SC10-65-H400 from parents and NILs but not from the recurrent parent. Segregation analysis showed that this marker was linked to Rh on chromosome 3L. Two bulks were then constructed by taking equal amount of DNA from 5 individuals of each group showing presence and absence of SC10-65-H400. The bulks were then screened using 700 primers. 7 products were found in resistant bulks and parents.

Asnaghi *et al* (2004) studied the presence of brown rust resistance gene

(Bru1) in cultivar R570 by segregation analysis in a large population of about 658 individuals. Amplified fragment length polymorphism (AFLP) and Bulked segregant analysis techniques were used to analyze a subset of this population and a detailed genetic map for resistance gene was developed using these techniques. Four hundred and forty three primer pairs were used and eight AFLP markers were identified at interval of 10 cM. The closest markers were located at 1.9 and 2.2 cM on each side of the gene.

Tiwari *et al* (2016) conducted a study to identify QTLs for salt tolerance in rice at the reproductive stage by conducting bulked segregant analysis of bi parental recombinant inbred lines. For formation of pools, to be used in BSA, 30 RILs were taken. The bulks and parents were then genotyped using 50K SNP chip and genomic regions that showed homogeneity for contrasting alleles of polymorphic SNPs in bulks were identified. A population derived from the cross CSR11 / M148 RIL was used for the study. 6,068 polymorphic SNPs were identified and 21 QTLs showed homogeneity in the bulks. The results were further analyzed using SSR markers.

Altinkut and Gozukirmizi (2003) performed a study to search microsatellite markers associated with water stress tolerance in wheat. They used bulked segregant analysis as an approach to identify the markers. They formed two DNA pools which were tolerant and sensitive. The pools were developed from a F_2 population on the basis of paraquat tolerance, leaf size and relative water content because these traits were related to water stress tolerance. Thirty five primer pairs were used for detection of microsatellite fragments that were present or absent in the pools as well as the parents. One fragment was seen present in tolerant parent and tolerant bulk. This marker could help in successful selection of water stress tolerant genotypes of wheat.

CHAPTER-III

MATERIAL AND METHODS

The materials used and procedures adopted during the course of investigation are discussed as under:

3.1 EXPERIMENTAL SITE

The experiment was conducted at rice experimental area, Department of Plant Breeding and Genetics, Punjab Agricultural University, Ludhiana during *kharif* season of the year 2017.

3.2 LOCATION AND CLIMATE

The experimental site is situated at latitude of 30˚54′ N and longitude of 75˚48′ E at a mean height of 247 meters above mean sea level in the trans-Gangetic agro climatic zone of India. This place is characterized by typical sub-tropical climate with hot and dry summer during April to June followed by hot and humid monsoon period during July to mid-September and cold winters during December and January. During summer, the maximum temperature lies between 40-45˚C and occasionally goes up to 47˚C, while the minimum temperature lies below 4˚C or less with frequent frosty spells during the month of December and January in winter. The area experiences an average rainfall of 700 mm mostly during the monsoon season and approximately 80% is received during the south-west monsoon period (July – September).

3.3 EXPERIMENTAL MATERIAL

The experimental material used for this study comprised of two F_2 populations from crosses PR121 X PR125 and PR121 X PR126, respectively with approximately 500 plants each.

3.4 EXPERIMENTAL DETAILS

Experiment No. 1

i) **Name of the experiment:** Anatomical studies of damaged grains in *Oryza sativa* cv. PR125.

ii) **Location of work:** Electron microscopy and nanotechnology lab, PAU, Ludhiana.

iii) **Methodology:** The variety possessing black pin point damage i.e. PR125, the normal cultivar which has no damage i.e. PR121 and the F_3 grains obtained by sowing F_2 generation of cross PR121 × PR125 were examined thoroughly under the stereo-microscope and scanning electron microscope to observe any kind of cell malformations as compared to normal grains.

Scanning electron microscopy

a) **Preparation of sample:** About 10 grains from each plant sample i.e. PR125, PR121 and F_3 seeds of cross PR121 × PR125 were selected and washed with tap water to remove the impurities. The grains were halved from the point of damage with a sterilized razor blade by viewing under stereo- microscope. A transverse section of the grains was also cut.

b) **Imaging under the Scanning Electron Microscope:** The samples were mounted on the SEM stub with the support of an aluminium stand. The stand allows the grain sample to sit on the stub at an angle that enabled a view of horizontal and transverse section of the grain surface. The specimens were mounted on a viewing stub with an adhesive tape and were then coated with gold (20-30nm) in a sputter coated. The stub was then placed in the Scanning electron microscope and photographs were taken in the Electron microscopy and nanotechnology lab of Punjab Agricultural University, Ludhiana.

iv) **Observations recorded:** Cell malformation or cell necrosis at the point of damage was recorded. The point of damage, cell malformation at point of damage and formation of starch granules at the point were viewed at 50X, 100X, 200X and 500X magnifications.

v) **Statistical Analysis:** Nil

Experiment No. 2

i) **Name of the experiment:** Phenotyping of F_2:F_3 seeds of cross PR121 × PR125 for damage in rice grains.

ii) **Location of work:** Rice experimental area, PAU, Ludhiana.

iii) **Methodology:** The following operations were done under this experiment:

a) **Sowing of nursery and transplanting**: Sowing was done in the last week of May and transplanting was done in first week of July, 2017. Approximately 500 F_2 plants of cross PR121 × PR125 and PR121 × PR126 were clonally replicated to generate a sufficient amount of F_2:F_3 seeds. The plants were sown in three replications. The two F_2 populations were named F_2-15 and F_2-17 respectively. The recommended package of practices was followed to raise the healthy crop.

b) **Harvesting**: F_3 seed was harvested from individual F_2 plant in all three clonal replicates in the last week of October.

c) **Threshing**: Threshing of paddy grains was done manually.

d) **Milling**: To further avoid any breakage in the grains, manual milling was done using sandpaper. For milling, 25 gram sample from seed of each F_2 plant was weighed in two replicates.

e) **Sorting of milled grains into damaged and undamaged grains**: The milled rice grains from each replicated sample were then sorted into damaged and undamaged grains manually. The grains were keenly observed for presence of any kind of damage.

iv) **Observations recorded:** Intensity of black pin point character was scored and data was recorded. The damaged grains were weighed separately. The percentage damage intensity was calculated by dividing the weight of damaged grains by total weight i.e. 25 grams multiplied by 100. Scoring for damage was also done on a scale of 1-5 to give rapid score to extent of damage.

$$\textbf{Per cent damage intensity per plant} = \frac{\textbf{Weight of damaged grains}}{\textbf{Total weight of sample (25 grams)}} \times \mathbf{100}$$

Scoring of data

- Scoring was done by making classes of percent damage intensity.
- For both the populations maximum and minimum percentage of damage was calculated and range was made accordingly.
- Scoring was done on a scale of 1-5 for both the F_2 populations.
- Damage count was also established. i.e number of plants that come under one score was calculated.

Scoring of plant population PR121 X PR125 and PR121 X PR126

Range of damage intensity	Score
<1.0	1
1.0-2.0	2
2.0-3.0	3
3.0-4.0	4
>4.0	5

The plants with <1% damage were given score 1 which represented the class of minimum damage. The plants with >4% damage were kept under score 5 which represented the class of maximum damage. The score 2, 3 and 4 were given to the plants with damage percentage ranging from 1-4%. Scoring of both the populations was done on the same scale.

v) Statistical Analysis: Chi square ($\chi 2$) test for inheritance was carried out to analyze the complexity of the black pinpoint character.

Chi square ($\chi 2$) was calculated using the formula:

$$\chi 2 = \frac{(O - E)^2}{E} \times 100$$

Experiment No. 3

i) Name of the experiment: Bulked segregant analysis to find out association of rice SSR markers with damage in rice grains.

ii) Location of work: Central Molecular Biology Laboratory, Department of Plant Breeding and Genetics, PAU, Ludhiana.

iii) Methodology:

a) Based on phenotypic analysis, DNA of 10 plants with extreme phenotypes was pooled to generate bulks.

b) Parental polymorphism was carried out using rice SSR (simple sequence repeats) markers.

c) Parental polymorphic markers were amplified in parents and bulks to identify the markers association with phenotype.

The following operations were carried out in this experiment:

a) Extraction of DNA: DNA was extracted using CTAB method. The procedure is described as follows:

1. Collection of plant samples from field.
2. About 5-7 gram of samples was taken and crushed using liquid nitrogen. The frozen powder was then transferred to a centrifuge tube containing CTAB extraction buffer (procedure shown in Table 3.4.3a).
3. The mixture was incubated for 20-30 mins at 65˚C for an hour in a water bath.
4. Equal volume of chloroform: isoamyl alcohol (24:1) was added.
5. Centrifugation at 12,000 rpm for 10 min.
6. The supernatant was transferred to a clean sterile tube and 0.8 ml volume of isopropyl alcohol cooled at 4˚C was added. Invert the tubes several times. DNA forms white cotton like precipitates and floats atop. The floating DNA was hooked out using a hooked Pasteur pipette or a wooden prick.
7. The hooked or pelleted DNA was transferred into a clean and sterile 2.0 ml tube.

The DNA was then washed with 1 ml 70% ethanol 2-3times.

8. Ethanol was decanted and the pellets were air dried for 30 min.
9. The DNA pellets were then suspended in100-200 µl TE buffer (procedure as in Table 3.4.3b).
10. DNA can then be stored at 4˚C for several months and at -20˚C for many years.

Table 3.4.3a: Preparation of cTAB

Chemical	500 ml	1000 ml
Cetrimide	10 g	20 g
Tris Base	6.057 g	12.114 g
NaCl	40.95 g	81.9 g
EDTA	3.722 g	7.445 g
B- mercaptoethanol	1.0 g	2.0 g

Table 3.4.3b: Preparation of TE Buffer

Component	100 ml	200 ml
Tris HCl	0.158 g	0.316 g
EDTA	0.037 g	0.74

b) **SSR Analysis:** Simple sequence repeats (SSR) markers were used for BSA and all the SSR were amplified using following PCR conditions.

1. The DNA template obtained after extraction was diluted and 2.0µl of dilute was taken in 0.5 ml eppendorf tube.
2. A mix of water, buffer, dNTPs, $MgCl_2$, forward and reverse primers and *Taq* polymerase was prepared as described in Table 3.4.3(c).
3. The mix was added to PCR plates containing DNA and plates were kept in thermal cycler.
4. Amplification was carried out for 35 cycles.
5. PCR products were then analyzed by Agarose gel electrophoresis.

Table 3.4.3c: Preparation of PCR mix

Component	Quantity (µl)
Buffer	4.0
$MgCl_2$	1.2

dNTPs	3.0
Forward Primer	1.0
Reverse Primer	1.0
Taq polymerase	0.2
DNA	2.5
Sterile Water	7.1

Table 3.4.3d: PCR temperature profile

Step I	94˚C	5 min	Initial denaturation
Step II	94˚C	1 min	Denaturation (30-35 cycles)
Step III	55˚C	1 min	Annealing
Step IV	72˚C	2 min	Primer extension
Step V			Repeat above steps
Step VI	72˚C	7 min	Completion of synthesis
Step VII	4˚C		Storage

c) **Agarose Gel Electrophoresis:** The samples were analyzed using agarose gel electrophoresis. The procedure is described as follows:

1. Firstly for preparation of gel, 3 g agarose was dissolved in 300 ml of 1X TBE buffer (Table 3.4.3(e)) for 0.1% gel and boiled in a microwave till fully dissolved and then cooled.
2. Ethidium Bromide was then added to gel for staining. It was added to obtain a final concentration of 0.5µg/ml buffer.
3. The agarose gel was then poured in the gel tray.
4. A comb was inserted before solidification of the gel. The gel was then allowed to solidify for 20-30 mins.
5. 1X TBE buffer was added to the gel tank to cover the gel. The solidified gel was then transferred to buffer tank and the combs removed.
6. DNA was loaded on the gel along with 1 Kb ladder.
7. The gel was allowed to run for 30-45minutes at 120mA.
8. After the gel was completely run, the images were taken using the gel doc system.

Table 3.4.3e: Preparation of 10% TBE Buffer

Components	1 litre	2 litre
Tris Base	109.02 g	218.04 g
Boric acid	55.6 g	111.2 g
EDTA	7.44 g	14.88 g

d) **Bulked Segregant Analysis:** The procedure is described as follows:

1. For bulked segregant analysis, the plants with extreme characteristics were chosen.
2. DNA from 10 plants of both extreme phenotypes i.e. (one with maximum damage and one with no or very less damage) was extracted using the cTAB method.
3. Two bulks were prepared one having less or no damage and other one having high damage intensity.
4. The bulks were then analyzed with the markers that were found to be polymorphic for the parents and results were seen using agarose gel electrophoresis.

iv) Observations to be recorded: Marker segregation in parents and bulks was recorded to find association of SSR marker(s) with phenotype.

v) Statistical Analysis: Nil

CHAPTER IV

RESULTS AND DISCUSSION

The results obtained in the present study have been presented and discussed experiment wise in this chapter.

4.1 Experiment I: Anatomical studies of damaged grains in *Oryza sativa* cv. PR125.

Cell damage refers to any kind of structural or functional impairment of cell constituents or cell organelles or any kind of stresses due to external as well as internal environment of the cell. This may occur due to physical, chemical, biological, nutritional, genetic or physiological factors. The damaged grains are the one in which the kernels or parts of kernel are internally damaged due to heat, moisture, microbe contamination (FSSAI 2011).

The black pinhead damage is a black dot like appearance on the surface of the grain which is clearly visible with naked eye. It is mostly seen on the upper surface of rice grain (Plate 1).

Damage at cellular level can be caused by microbial contamination, physiological disorders, biochemical reactions, structural impairment and certain factors like moisture content, temperature and storage conditions.

The grains were examined for damage at cellular level using scanning electron microscopy. The grains were cut by viewing under the stereo microscope for better view. Under stereo microscope the point of damage could be easily seen and grain was easily cut at the point of damage. Both damaged and undamaged grains were examined to compare the cell formation in both types of grains. SEM analysis on both the parents as well as the population was carried out. The undamaged parental variety PR121 was kept as control for comparison between damaged and undamaged grains (Plate 2). Before viewing the grains were washed and prepared for taking pictures. Pictures were taken at 50, 100, 200 and 500 X magnifications to have a better and deeper view of the grain constituents. The grains were studied at 15KV.

4.1.1 Cell damage

The rice grains are composed of starch, protein and lipids which affect the cooking and eating quality of rice grains (Zhou *et al* 2002). The grains showed the differential structure of starch granules in damaged grains as compared to the undamaged ones. The starch granules in the undamaged grain were tightly packed with no deterioration of starch structure. Amylose and amylopectin which are the

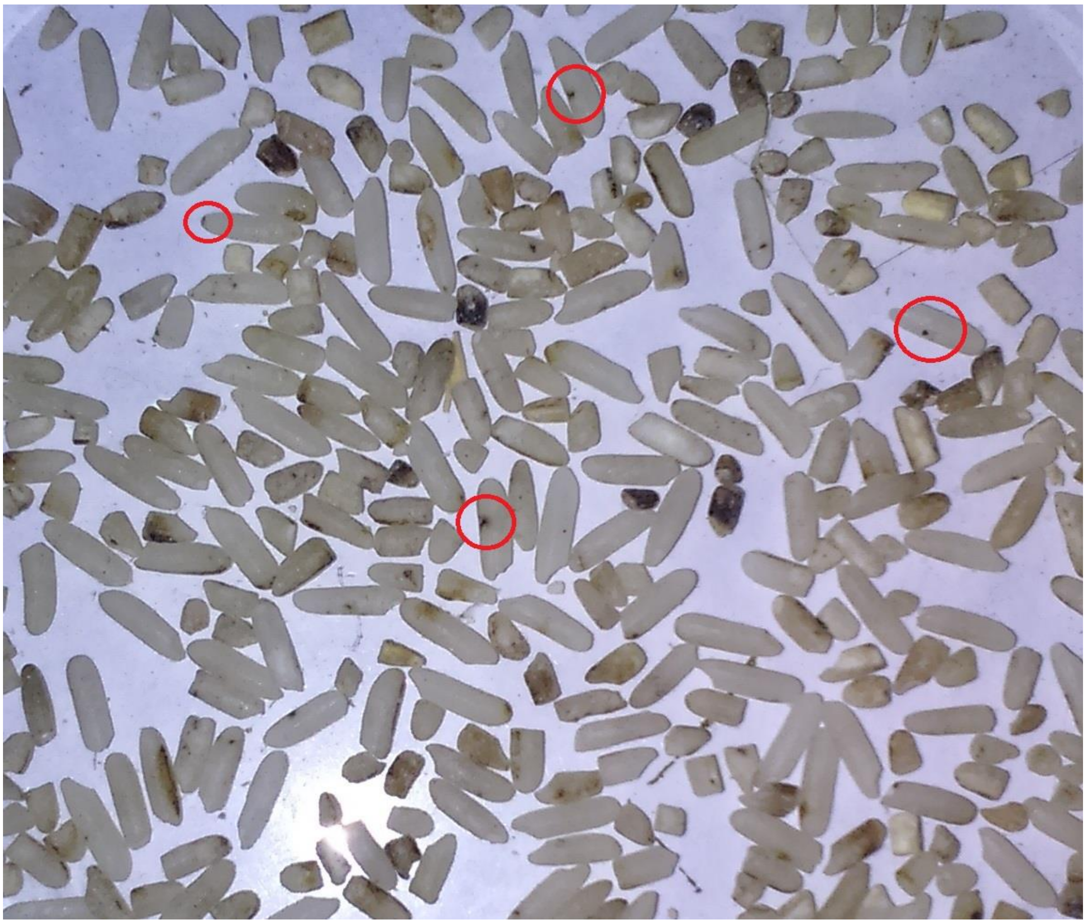

Plate 1: Point of damage (black pinhead) as seen on the damaged grains of PR 125.

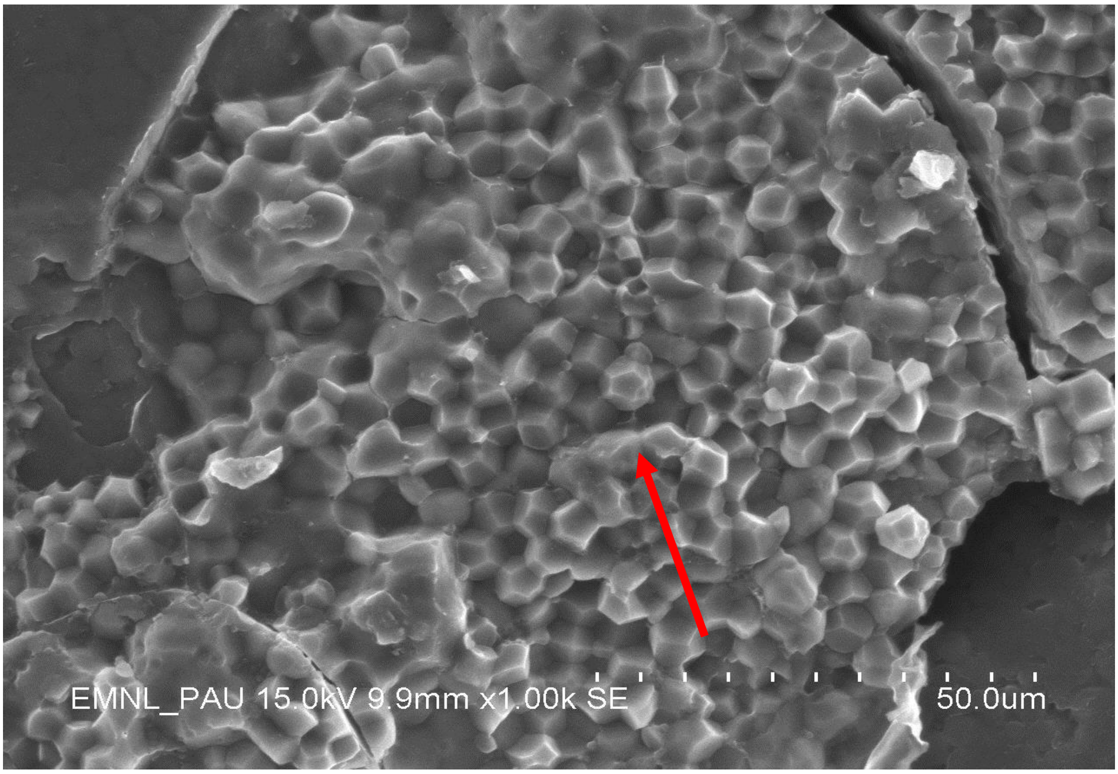

Plate 2: Image obtained under scanning electron microscope showing normal (compact) starch granules of PR121 taken as control.

main constituents and structure forming components of starch were well arranged. The normal grains showed the normal granular structure when seen under electron microscope. The grain was cut transversally which showed normal structure of starch granules in the grain.

On the contrary the starch granules in the damaged grains were seen to be distorted. The starch granules were found to be loosely packed (Plate 3). Similar results were observed for both the populations as well (Plate 4 and Plate 5). There was no sign of fungal contamination both in undamaged and damaged grains. As in PAU 201, the grains in PR125 were also observed to break easily during milling hence reducing the head rice percentage and lowering the quality and consumer acceptability of rice grains. (Siruguri *et al* 2012).

The damage in current study was not found to be associated with any kind of fungal contamination. However, similar studies conducted on Phalguna rice variety showed conclusively that black discoloration was the result of fungal invasion. In the current study the damaged grains appeared to be more brittle and break easily at point of damage. Similar results were seen in case of Phalguna variety where the grains were more brittle and appeared to adversely affect the milling quality of the variety. In fact, the milling quality of Phalguna paddy was poor and further deteriorated when it was affected by black tip (Bhat *et al* 1982).

The transverse section was cut through the point of damage by viewing under stereo microscope. The oblique and transverse sections were cut through the point of damage which demonstrated that the black point was not confined to surface only (Plate 6). It was embedded inside the grain. It created a depression from surface to inside of the grains.

Distortion of starch structure may be attributed to variation in amylose and amylopectin ratio which can be greatly affected by genetic, environmental and agronomic factors. (Lawal *et al* 2011). Distortion of starch may also be attributed to some physiological factors that may cause damage to starch structure.

4.2 Experiment II: Phenotyping of F_2:F_3 seeds of cross PR121×PR125 for damage in rice grains.

4.2.1 Per cent damage intensity

The rice grains were milled manually using sand paper and sorted into damaged and undamaged grains. The weight of damaged grains was taken for each plant sample in two replications of 25 grams each.

Table 4.2.1(a): Weight and percent damage intensity of damaged grains (grams) per plant in cross (PR121 X PR125)

Plant number	Weight of damaged grains/ sample (R-1)	Weight of damaged grains/ sample (R-2)	Mean weight (g)	%damage intensity (R_1)	% damage intensity (R_2)	Mean damage intensity (%)
1	0.32	0.24	0.3	1.3	1.0	1.1
2	0.49	0.39	0.4	2.0	1.6	1.8
3	0.14	0.43	0.3	0.6	1.7	1.1
4	0.23	0.4	0.3	0.9	1.6	1.3
5	0.41	0.39	0.4	1.6	1.6	1.6
6	0.16	0.26	0.2	0.6	1.0	0.8
7	0.64	0.63	0.6	2.6	2.5	2.5
8	0.11	0.18	0.1	0.4	0.7	0.6
9	0.29	0.67	0.5	1.2	2.7	1.9
10	0.35	0.17	0.3	1.4	0.7	1.0
11	1.47	0.69	1.1	5.9	2.8	4.3
12	0.11	0.55	0.3	0.4	2.2	1.3
13	0.27	0.45	0.4	1.1	1.8	1.4
14	0.24	0.34	0.3	1.0	1.4	1.2
15	0.6	1.2	0.9	2.4	4.8	3.6
16	0.41	0.55	0.5	1.6	2.2	1.9
17	0.35	0.24	0.3	1.4	1.0	1.2
18	0.7	0.4	0.6	2.8	1.6	2.2
19	0.44	0.48	0.5	1.8	1.9	1.8
20	0.75	0.42	0.6	3.0	1.7	2.3
21	1.05	0.57	0.8	4.2	2.3	3.2
22	0.8	0.65	0.7	3.2	2.6	2.9
23	0.14	0.31	0.2	0.6	1.2	0.9
24	1.04	0.98	1.0	4.2	3.9	4.0
25	0.82	0.67	0.7	3.3	2.7	3.0
26	1.27	1.95	1.6	5.1	7.8	6.4
27	1.39	1.13	1.3	5.6	4.5	5.0
28	1.16	0.64	0.9	4.6	2.6	3.6

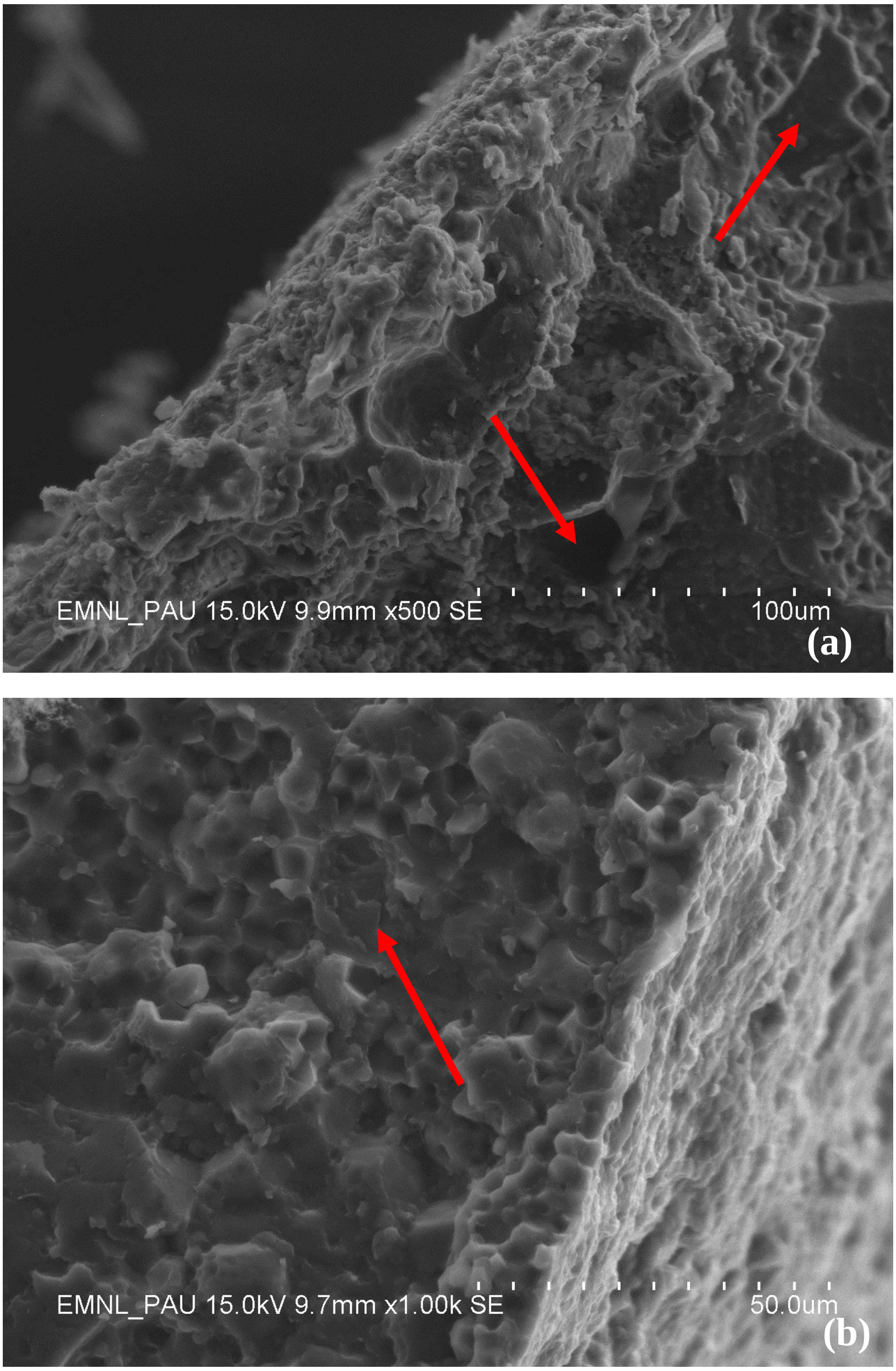

Plate 3: Comparison of damaged and normal starch granules of PR125 as obtained under scanning electron microscope (a) damaged starch granules showing loose packing of granules and empty voids, (b) normal granules showing compact structure.

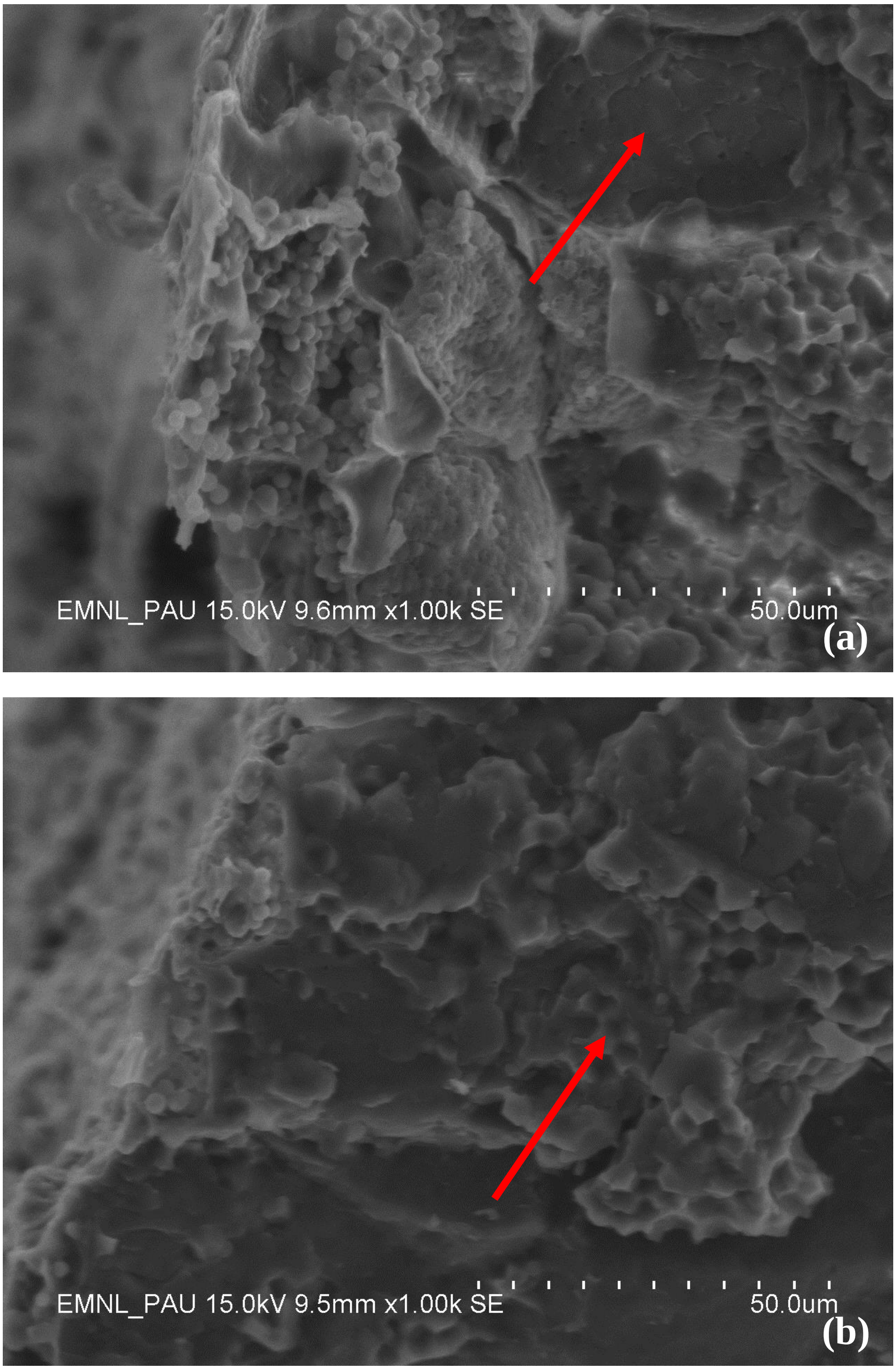

Plate 4: Comparison of damaged and normal starch granules of cross PR121 × PR125 as obtained under scanning electron microscope (a) damaged starch granules showing loose packing of granules and empty voids, (b) normal granules showing compact starch granules.

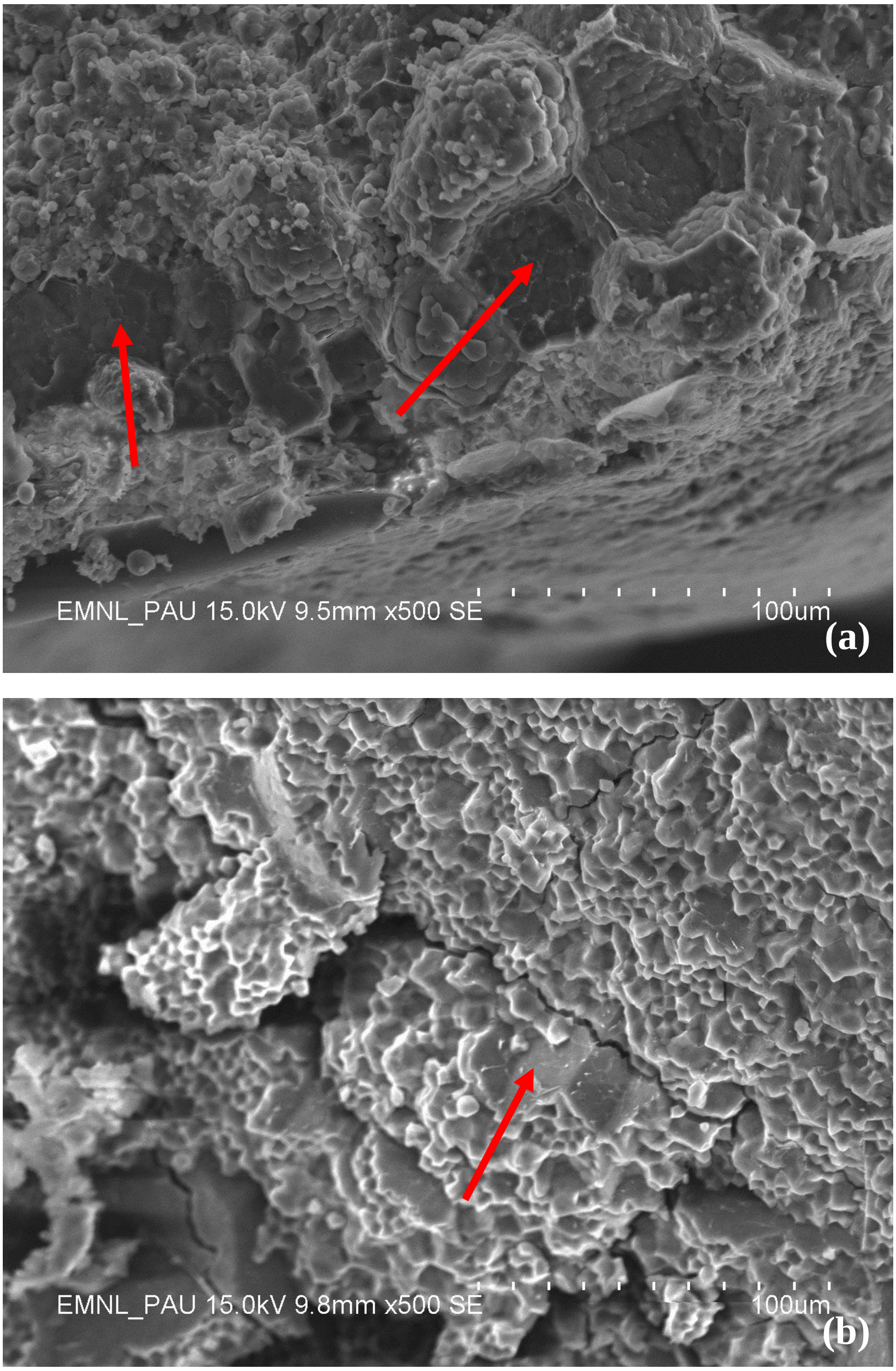

Plate 5: Comparison of damaged and normal starch granules of cross PR121 × PR126 as obtained under scanning electron microscope (a) damaged starch granules showing loose packing of granules and empty voids, (b) normal granules showing compact starch granules.

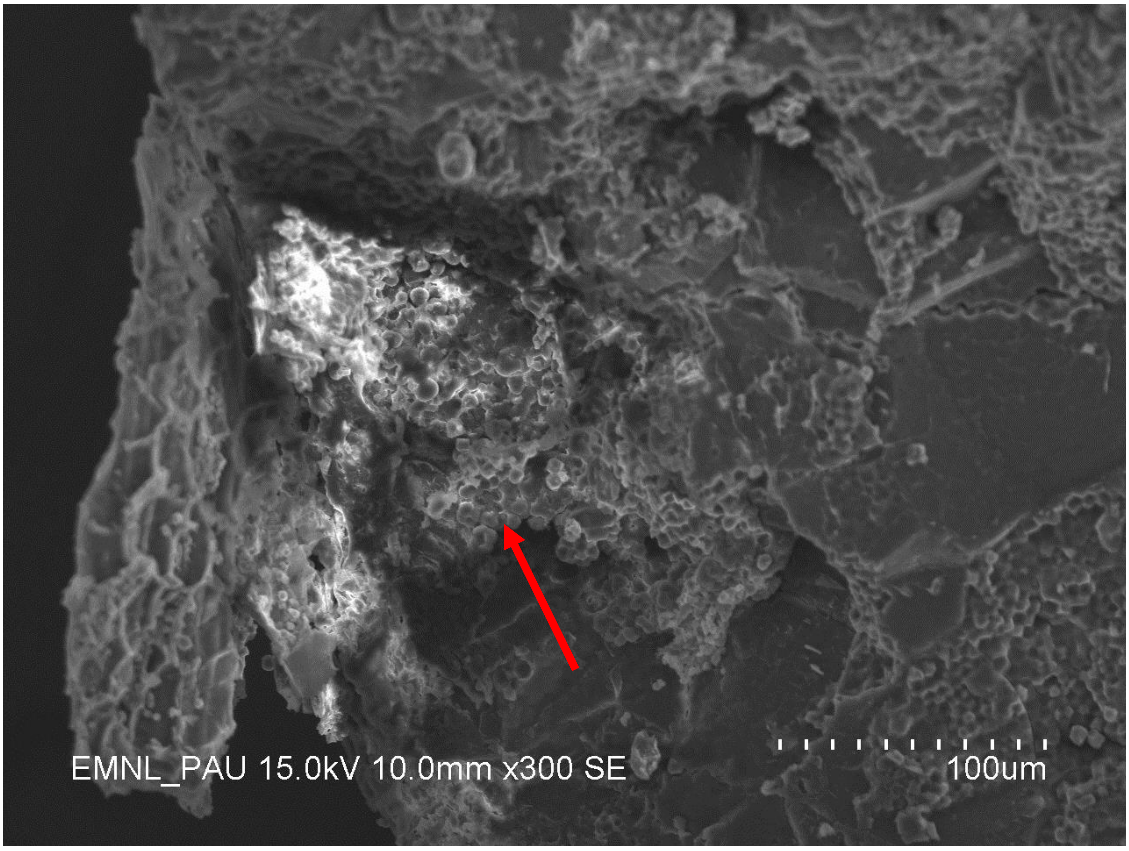

Plate 6: Depression created in the grain as a result of black pinhead damage

Plant number	**Weight of damaged grains/ sample (R-1)**	**Weight of damaged grains/ sample (R-2)**	**Mean weight (g)**	**%damage intensity (R_1)**	**% damage intensity (R_2)**	**Mean damage intensity (%)**
29	0.86	0.45	0.7	3.4	1.8	2.6
30	0.45	0.47	0.5	1.8	1.9	1.8
31	0.56	0.54	0.6	2.2	2.2	2.2
32	0.59	0.61	0.6	2.4	2.4	2.4
33	0.38	0.4	0.4	1.5	1.6	1.6
34	0.47	0.49	0.5	1.9	2.0	1.9
35	0.35	0.62	0.5	1.4	2.5	1.9
36	0.32	0.48	0.4	1.3	1.9	1.6
37	0.61	0.56	0.6	2.4	2.2	2.3
38	0.68	0.27	0.5	2.7	1.1	1.9
39	0.52	0.45	0.5	2.1	1.8	1.9
40	0.58	0.53	0.6	2.3	2.1	2.2
41	0.63	0.43	0.5	2.5	1.7	2.1
42	0.51	0.32	0.4	2.0	1.3	1.7
43	0.39	0.58	0.5	1.6	2.3	1.9
44	0.37	0.52	0.4	1.5	2.1	1.8
45	0.38	0.48	0.4	1.5	1.9	1.7
46	0.39	0.2	0.3	1.6	0.8	1.2
47	0.5	0.88	0.7	2.0	3.5	2.8
48	0.44	0.55	0.5	1.8	2.2	2.0
49	0.31	0.42	0.4	1.2	1.7	1.5
50	0.44	0.52	0.5	1.8	2.1	1.9
51	0.58	0.68	0.6	2.3	2.7	2.5
52	0.52	0.59	0.6	2.1	2.4	2.2
53	0.43	0.23	0.3	1.7	0.9	1.3
54	0.69	0.85	0.8	2.8	3.4	3.1
55	0.83	1.2	1.0	3.3	4.8	4.1
56	0.8	1.34	1.1	3.2	5.4	4.3
57	0.63	0.67	0.7	2.5	2.7	2.6

Plant number	Weight of damaged grains/ sample (R-1)	Weight of damaged grains/ sample (R-2)	Mean weight (g)	%damage intensity (R_1)	% damage intensity (R_2)	Mean damage intensity (%)
58	0.68	0.58	0.6	2.7	2.3	2.5
59	0.57	0.49	0.5	2.3	2.0	2.1
60	0.37	0.31	0.3	1.5	1.2	1.4
61	0.57	0.49	0.5	2.3	2.0	2.1
62	0.87	0.83	0.9	3.5	3.3	3.4
63	0.64	0.62	0.6	2.6	2.5	2.5
64	0.81	0.75	0.8	3.2	3.0	3.1
65	1.17	1.02	1.1	4.7	4.1	4.4
66	0.64	0.52	0.6	2.6	2.1	2.3
67	0.79	0.54	0.7	3.2	2.2	2.7
68	0.35	0.26	0.3	1.4	1.0	1.2
69	0.4	0.32	0.4	1.6	1.3	1.4
70	0.47	0.49	0.5	1.9	2.0	1.9
71	0.59	0.49	0.5	2.4	2.0	2.2
72	0.56	0.43	0.5	2.2	1.7	2.0
73	1.03	0.64	0.8	4.1	2.6	3.3
74	0.71	0.16	0.4	2.8	0.6	1.7
75	0.6	0.59	0.6	2.4	2.4	2.4
76	0.52	0.61	0.6	2.1	2.4	2.3
77	0.32	0.52	0.4	1.3	2.1	1.7
78	1.74	1.85	1.8	7.0	7.4	7.2
79	0.76	1.29	1.0	3.0	5.2	4.1
80	0.4	0.33	0.4	1.6	1.3	1.5
81	1.38	0.55	1.0	5.5	2.2	3.9
82	1.15	0.76	1.0	4.6	3.0	3.8
83	0.76	0.66	0.7	3.0	2.6	2.8
84	0.23	0.51	0.4	0.9	2.0	1.5
85	0.33	0.56	0.4	1.3	2.2	1.8
86	0.4	0.37	0.4	1.6	1.5	1.5
87	0.45	0.32	0.4	1.8	1.3	1.5

Plant number	**Weight of damaged grains/ sample (R-1)**	**Weight of damaged grains/ sample (R-2)**	**Mean weight (g)**	**%damage intensity (R_1)**	**% damage intensity (R_2)**	**Mean damage intensity (%)**
88	0.24	0.45	0.3	1.0	1.8	1.4
89	0.35	0.29	0.3	1.4	1.2	1.3
90	0.39	0.32	0.4	1.6	1.3	1.4
91	0.49	0.35	0.4	2.0	1.4	1.7
92	0.39	0.33	0.4	1.6	1.3	1.4
93	0.78	0.61	0.7	3.1	2.4	2.8
94	0.99	0.64	0.8	4.0	2.6	3.3
95	0.18	0.25	0.2	0.7	1.0	0.9
96	1.16	1.41	1.3	4.6	5.6	5.1
97	0.6	0.47	0.5	2.4	1.9	2.1
98	0.53	0.59	0.6	2.1	2.4	2.2
99	0.17	0.35	0.3	0.7	1.4	1.0
100	0.27	0.34	0.3	1.1	1.4	1.2
101	0.29	0.46	0.4	1.2	1.8	1.5
102	0.58	0.64	0.6	2.3	2.6	2.4
103	0.62	0.57	0.6	2.5	2.3	2.4
104	0.72	0.69	0.7	2.9	2.8	2.8
105	0.21	0.24	0.2	0.8	1.0	0.9
106	0.58	0.54	0.6	2.3	2.2	2.2
107	0.34	0.29	0.3	1.4	1.2	1.3
108	0.44	0.3	0.4	1.8	1.2	1.5
109	0.79	0.44	0.6	3.2	1.8	2.5
110	0.74	0.54	0.6	3.0	2.2	2.6
111	0.22	0.18	0.2	0.9	0.7	0.8
112	0.71	0.65	0.7	2.8	2.6	2.7
113	0.59	0.41	0.5	2.4	1.6	2.0
114	0.45	0.37	0.4	1.8	1.5	1.6
115	0.25	0.23	0.2	1.0	0.9	1.0
116	0.8	0.51	0.7	3.2	2.0	2.6
117	0.34	0.42	0.4	1.4	1.7	1.5

Plant number	Weight of damaged grains/ sample (R-1)	Weight of damaged grains/ sample (R-2)	Mean weight (g)	%damage intensity (R_1)	% damage intensity (R_2)	Mean damage intensity (%)
118	1.08	1.25	1.2	4.3	5.0	4.7
119	0.31	0.26	0.3	1.2	1.0	1.1
120	1.09	0.56	0.8	4.4	2.2	3.3
121	0.6	0.53	0.6	2.4	2.1	2.3
122	0.67	1.87	1.3	2.7	7.5	5.1
123	0.77	0.62	0.7	3.1	2.5	2.8
124	0.42	0.37	0.4	1.7	1.5	1.6
125	1.47	1.09	1.3	5.9	4.4	5.1
126	0.85	0.4	0.6	3.4	1.6	2.5
127	1.06	0.62	0.8	4.2	2.5	3.4
128	0.97	0.99	1.0	3.9	4.0	3.9
129	0.54	0.5	0.5	2.2	2.0	2.1
130	0.37	0.35	0.4	1.5	1.4	1.4
131	0.8	0.93	0.9	3.2	3.7	3.5
132	0.42	0.43	0.4	1.7	1.7	1.7
133	0.38	0.4	0.4	1.5	1.6	1.6
134	0.5	0.35	0.4	2.0	1.4	1.7
135	0.58	0.71	0.6	2.3	2.8	2.6
136	0.55	0.78	0.7	2.2	3.1	2.7
137	0.4	0.61	0.5	1.6	2.4	2.0
138	0.42	0.2	0.3	1.7	0.8	1.2
139	0.55	0.27	0.4	2.2	1.1	1.6
140	0.29	0.49	0.4	1.2	2.0	1.6
141	0.49	0.62	0.6	2.0	2.5	2.2
142	0.29	0.24	0.3	1.2	1.0	1.1
143	0.37	0.26	0.3	1.5	1.0	1.3
144	0.55	0.42	0.5	2.2	1.7	1.9
145	0.38	0.26	0.3	1.5	1.0	1.3
146	0.13	0.28	0.2	0.5	1.1	0.8
147	0.63	0.88	0.8	2.5	3.5	3.0

Plant number	**Weight of damaged grains/ sample (R-1)**	**Weight of damaged grains/ sample (R-2)**	**Mean weight (g)**	**%damage intensity (R_1)**	**% damage intensity (R_2)**	**Mean damage intensity (%)**
148	0.43	0.54	0.5	1.7	2.2	1.9
149	0.43	0.63	0.5	1.7	2.5	2.1
150	0.71	0.45	0.6	2.8	1.8	2.3
151	0.81	0.36	0.6	3.2	1.4	2.3
152	0.91	0.29	0.6	3.6	1.2	2.4
153	0.94	0.73	0.8	3.8	2.9	3.3
154	0.13	0.1	0.1	0.5	0.4	0.5
155	0.17	0.49	0.3	0.7	2.0	1.3
156	0.21	0.17	0.2	0.8	0.7	0.8
157	0.16	0.26	0.2	0.6	1.0	0.8
158	0.35	0.46	0.4	1.4	1.8	1.6
159	0.28	0.42	0.4	1.1	1.7	1.4
160	0.73	0.99	0.9	2.9	4.0	3.4
161	0.16	0.36	0.3	0.6	1.4	1.0
162	0.15	0.2	0.2	0.6	0.8	0.7
163	0.49	0.35	0.4	2.0	1.4	1.7
164	0.59	0.37	0.5	2.4	1.5	1.9
165	0.69	0.54	0.6	2.8	2.2	2.5
166	0.53	0.37	0.5	2.1	1.5	1.8
167	0.27	0.31	0.3	1.1	1.2	1.2
168	0.2	0.31	0.3	0.8	1.2	1.0
169	0.28	0.3	0.3	1.1	1.2	1.2
170	1.15	0.35	0.8	4.6	1.4	3.0
171	0.85	0.4	0.6	3.4	1.6	2.5
172	0.36	0.27	0.3	1.4	1.1	1.3
173	0.23	0.31	0.3	0.9	1.2	1.1
174	0.57	0.39	0.5	2.3	1.6	1.9
175	0.36	0.48	0.4	1.4	1.9	1.7
176	0.4	0.45	0.4	1.6	1.8	1.7
177	0.2	0.12	0.2	0.8	0.5	0.6

Plant number	Weight of damaged grains/ sample (R-1)	Weight of damaged grains/ sample (R-2)	Mean weight (g)	%damage intensity (R_1)	% damage intensity (R_2)	Mean damage intensity (%)
178	0.12	0.18	0.2	0.5	0.7	0.6
179	0.4	0.35	0.4	1.6	1.4	1.5
180	0.28	0.34	0.3	1.1	1.4	1.2
181	0.2	0.17	0.2	0.8	0.7	0.7
182	0.3	0.25	0.3	1.2	1.0	1.1
183	0.83	0.31	0.6	3.3	1.2	2.3
184	0.39	0.55	0.5	1.6	2.2	1.9
185	0.29	0.49	0.4	1.2	2.0	1.6
186	0.38	0.96	0.7	1.5	3.8	2.7
187	0.42	0.3	0.4	1.7	1.2	1.4
188	0.21	0.63	0.4	0.8	2.5	1.7
189	0.24	0.5	0.4	1.0	2.0	1.5
190	0.31	0.54	0.4	1.2	2.2	1.7
191	0.37	0.6	0.5	1.5	2.4	1.9
192	0.41	0.67	0.5	1.6	2.7	2.2
193	0.49	0.46	0.5	2.0	1.8	1.9
194	0.45	0.5	0.5	1.8	2.0	1.9
195	0.4	0.52	0.5	1.6	2.1	1.8
196	0.58	0.25	0.4	2.3	1.0	1.7
197	0.68	0.8	0.7	2.7	3.2	3.0
198	0.62	0.7	0.7	2.5	2.8	2.6
199	0.32	0.62	0.5	1.3	2.5	1.9
200	0.25	0.66	0.5	1.0	2.6	1.8
201	0.2	0.38	0.3	0.8	1.5	1.2
202	0.32	0.55	0.4	1.3	2.2	1.7
203	0.54	0.36	0.5	2.2	1.4	1.8
204	1.19	0.57	0.9	4.8	2.3	3.5
205	0.38	0.25	0.3	1.5	1.0	1.3
206	0.52	0.29	0.4	2.1	1.2	1.6
207	0.58	0.47	0.5	2.3	1.9	2.1

Plant number	Weight of damaged grains/ sample (R-1)	Weight of damaged grains/ sample (R-2)	Mean weight (g)	%damage intensity (R_1)	% damage intensity (R_2)	Mean damage intensity (%)
208	0.31	0.46	0.4	1.2	1.8	1.5
209	0.27	0.23	0.3	1.1	0.9	1.0
210	0.32	0.46	0.4	1.3	1.8	1.6
211	0.43	0.38	0.4	1.7	1.5	1.6
212	0.31	0.23	0.3	1.2	0.9	1.1
213	0.55	0.46	0.5	2.2	1.8	2.0
214	0.48	0.6	0.5	1.9	2.4	2.2
215	0.52	0.4	0.5	2.1	1.6	1.8
216	0.49	0.38	0.4	2.0	1.5	1.7
217	0.58	0.47	0.5	2.3	1.9	2.1
218	0.45	0.27	0.4	1.8	1.1	1.4
219	0.31	0.4	0.4	1.2	1.6	1.4
220	0.73	0.4	0.6	2.9	1.6	2.3
221	0.5	0.4	0.5	2.0	1.6	1.8
222	0.35	0.2	0.3	1.4	0.8	1.1
223	0.23	0.51	0.4	0.9	2.0	1.5
224	0.26	0.48	0.4	1.0	1.9	1.5
225	0.4	0.48	0.4	1.6	1.9	1.8
226	0.55	0.51	0.5	2.2	2.0	2.1
227	0.52	0.48	0.5	2.1	1.9	2.0
228	0.44	0.55	0.5	1.8	2.2	2.0
229	0.31	0.46	0.4	1.2	1.8	1.5
230	0.48	0.63	0.6	1.9	2.5	2.2
231	0.81	0.79	0.8	3.2	3.2	3.2
232	0.49	0.72	0.6	2.0	2.9	2.4
233	0.54	0.45	0.5	2.2	1.8	2.0
234	0.64	0.62	0.6	2.6	2.5	2.5
235	0.78	0.59	0.7	3.1	2.4	2.7
236	0.51	0.46	0.5	2.0	1.8	1.9
237	0.42	0.43	0.4	1.7	1.7	1.7

Plant number	**Weight of damaged grains/ sample (R-1)**	**Weight of damaged grains/ sample (R-2)**	**Mean weight (g)**	**%damage intensity (R_1)**	**% damage intensity (R_2)**	**Mean damage intensity (%)**
238	0.6	0.54	0.6	2.4	2.2	2.3
239	0.32	0.41	0.4	1.3	1.6	1.5
240	0.44	0.75	0.6	1.8	3.0	2.4
241	0.63	0.44	0.5	2.5	1.8	2.1
242	0.75	0.58	0.7	3.0	2.3	2.7
243	0.35	0.5	0.4	1.4	2.0	1.7
244	0.43	0.62	0.5	1.7	2.5	2.1
245	0.6	0.21	0.4	2.4	0.8	1.6
246	0.71	0.62	0.7	2.8	2.5	2.7
247	0.62	0.85	0.7	2.5	3.4	2.9
248	0.64	0.26	0.5	2.6	1.0	1.8
249	0.37	0.42	0.4	1.5	1.7	1.6
250	0.41	0.5	0.5	1.6	2.0	1.8
251	0.5	0.42	0.5	2.0	1.7	1.8
252	0.27	0.25	0.3	1.1	1.0	1.0
253	0.3	0.27	0.3	1.2	1.1	1.1
254	0.29	0.31	0.3	1.2	1.2	1.2
255	0.33	0.64	0.5	1.3	2.6	1.9
256	0.53	0.52	0.5	2.1	2.1	2.1

A total of 256 plants of the F_2 cross PR121 X PR125 were sown and multiplied to obtain a sufficient amount of seed. The weight of damaged grains was recorded in duplicate. The weight was taken using an electronic weighing balance. The samples were collected randomly. Two samples (R_1 and R_2) of 25 gram each were taken from one plant and milled and sorted into damaged and normal grains. Out of total 256 plants, 17 had damage more than or equal to 1.0 gram with 1.8 grams being the highest level of damage present in the population. The least damage was found to be 0.1 grams which was shown by only two plants (Table 4.2.1 (a)).

Percent damage intensity for F_3 grains of cross PR121 X PR125 were calculated per plant. Mean value for both the replications was taken to know the maximum and minimum value of per cent damage. Range of average per cent damage intensity was 0.5-7.2 (Table 4.2.1 (a)). In an earlier observation, the percent damage intensity including discolored and black pinpoint damage in PAU 201 was in the range of 3.1-12.25 per cent (Siruguri *et al* 2012). The mean and range of number of grains with black pinhead damage was recorded for both the replications in which data was taken. For the cross PR121 X PR125 mean damage of black pinhead was recorded to be 0.5172 with range of 0.11-1.79 in F_3 progenies (Table 4.2.1b, Figure 4.2.1a).

Table 4.2.1b: Mean and range of black pinhead damage (in grams) in cross PR121 X PR125:

Variable	**N**	**Mean ± SE**	**Range**
Replication 1	256	0.5244 ± 0.0171	0.11 – 1.74
Replication 2	256	0.5101 ± 0.0167	0.10 – 1.95
Mean	256	0.5172 ± 0.0151	0.11 – 1.79

The bell shaped curve on the graph represents the normal distribution of the black pinhead damage in cross PR121 X PR125 (Figure 4.2.1a).

The similar observations were also recorded for cross PR121 X PR126.

Only two out of total 255 plants had the damaged grains more than or equal to 1.0 gram (Table 4.2.1(c)). The range of black pinhead damage in cross PR121 X PR126 was observed to be 0.1-1.2 (Table 4.2.1d).

Maximum value of percent intensity of black pinhead damage was recorded to be 4.9 % and the minimum percent intensity was recorded to be 0.5%.

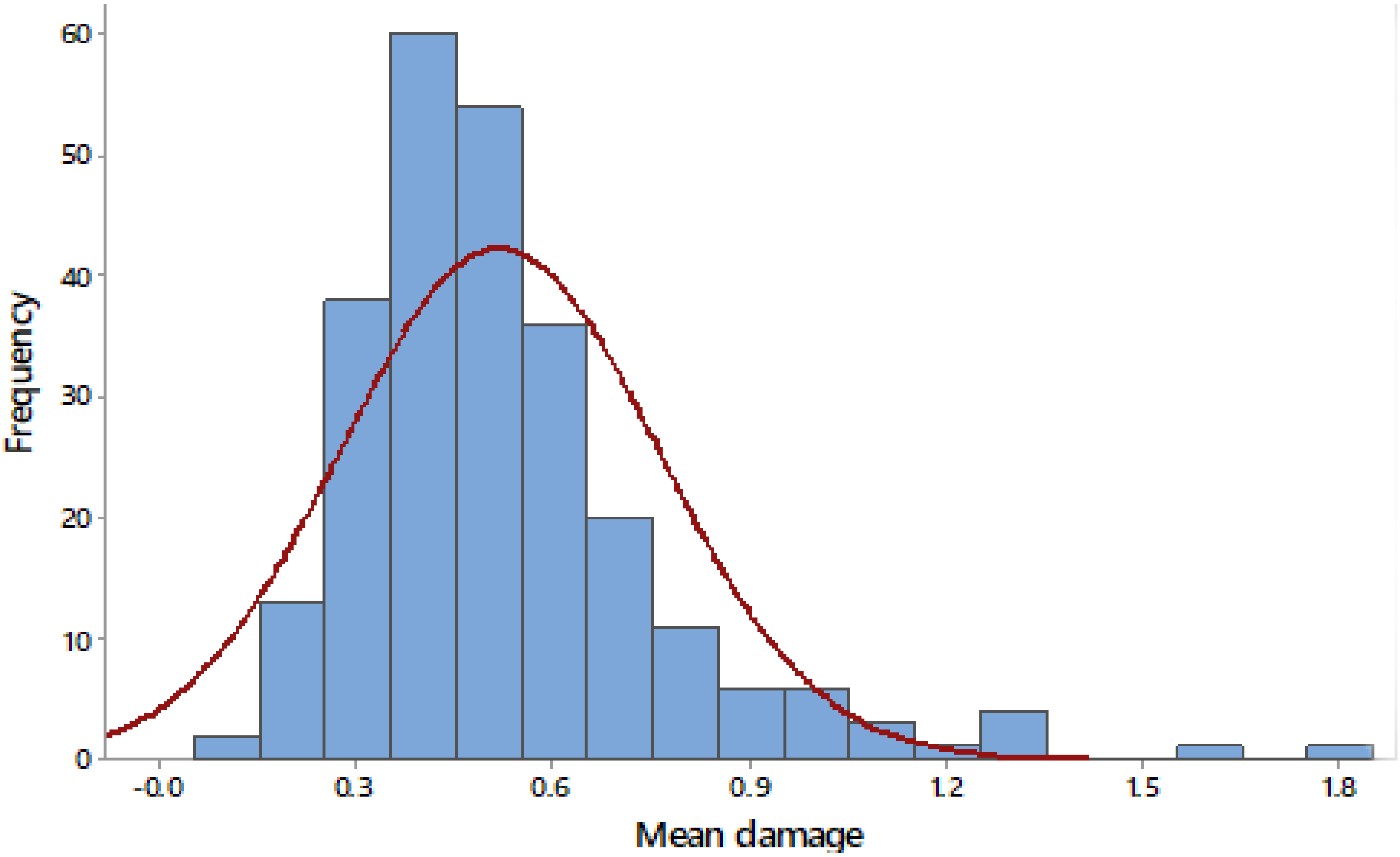

Figure 4.2.1a: Normal distribution of the black pinhead damage in cross PR121 X PR125.

Table 4.2.1(c): Weight and percent damage intensity of black pinhead damage per plant in cross PR121 X PR126

Plant number	Weight of damaged grains/ sample (R-1)	Weight of damaged grains/ sample (R-2)	Mean weight in grams	Percent damage intensity R1	Percent damage intensity R2	Mean damage intensity (%)
1	0.23	0.25	0.2	0.9	1.0	1.0
2	0.31	0.34	0.3	1.2	1.4	1.3
3	0.33	0.28	0.3	1.3	1.1	1.2
4	0.31	0.2	0.3	1.2	0.8	1.0
5	0.28	0.4	0.3	1.1	1.6	1.4
6	0.38	0.36	0.4	1.5	1.4	1.5
7	0.28	0.31	0.3	1.1	1.2	1.2
8	0.51	0.59	0.6	2.0	2.4	2.2
9	0.28	0.22	0.3	1.1	0.9	1.0
10	0.11	0.12	0.1	0.4	0.5	0.5
11	0.3	0.44	0.4	1.2	1.8	1.5
12	0.39	0.45	0.4	1.6	1.8	1.7
13	0.7	0.25	0.5	2.8	1.0	1.9
14	0.51	0.28	0.4	2.0	1.1	1.6
15	0.38	0.45	0.4	1.5	1.8	1.7
16	0.34	0.47	0.4	1.4	1.9	1.6
17	0.34	0.38	0.4	1.4	1.5	1.4
18	0.34	0.56	0.5	1.4	2.2	1.8
19	0.73	0.76	0.7	2.9	3.0	3.0
20	0.72	0.83	0.8	2.9	3.3	3.1
21	0.74	0.6	0.7	3.0	2.4	2.7
22	0.48	0.88	0.7	1.9	3.5	2.7
23	0.35	0.37	0.4	1.4	1.5	1.4
24	0.73	0.42	0.6	2.9	1.7	2.3
25	0.54	0.6	0.6	2.2	2.4	2.3

Plant number	Weight of damaged grains/ sample (R-1)	Weight of damaged grains/ sample (R-2)	Mean weight in grams	Percent damage intensity R1	Percent damage intensity R2	Mean damage intensity (%)
26	0.35	0.34	0.3	1.4	1.4	1.4
27	0.49	0.51	0.5	2.0	2.0	2.0
28	0.6	0.65	0.6	2.4	2.6	2.5
29	0.36	0.45	0.4	1.4	1.8	1.6
30	0.36	0.38	0.4	1.4	1.5	1.5
31	0.36	0.39	0.4	1.4	1.6	1.5
32	0.36	0.3	0.3	1.4	1.2	1.3
33	0.45	0.47	0.5	1.8	1.9	1.8
34	0.54	0.52	0.5	2.2	2.1	2.1
35	0.86	0.84	0.9	3.4	3.4	3.4
36	0.39	0.26	0.3	1.6	1.0	1.3
37	0.28	0.3	0.3	1.1	1.2	1.2
38	0.2	0.22	0.2	0.8	0.9	0.8
39	0.16	0.2	0.2	0.6	0.8	0.7
40	0.12	0.26	0.2	0.5	1.0	0.8
41	0.43	0.6	0.5	1.7	2.4	2.1
42	0.35	0.45	0.4	1.4	1.8	1.6
43	0.74	0.88	0.8	3.0	3.5	3.2
44	0.34	0.3	0.3	1.4	1.2	1.3
45	0.43	0.45	0.4	1.7	1.8	1.8
46	0.29	0.49	0.4	1.2	2.0	1.6
47	0.46	0.48	0.5	1.8	1.9	1.9
48	0.31	0.4	0.4	1.2	1.6	1.4
49	0.38	0.41	0.4	1.5	1.6	1.6
50	0.27	0.34	0.3	1.1	1.4	1.2
51	0.34	0.38	0.4	1.4	1.5	1.4
52	0.56	0.44	0.5	2.2	1.8	2.0
53	0.79	0.5	0.6	3.2	2.0	2.6

Plant number	**Weight of damaged grains/ sample (R-1)**	**Weight of damaged grains/ sample (R-2)**	**Mean weight in grams**	**Percent damage intensity R1**	**Percent damage intensity R2**	**Mean damage intensity (%)**
54	0.34	0.64	0.5	1.4	2.6	2.0
55	0.47	0.61	0.5	1.9	2.4	2.2
56	0.6	0.58	0.6	2.4	2.3	2.4
57	0.53	0.55	0.5	2.1	2.2	2.2
58	0.37	0.31	0.3	1.5	1.2	1.4
59	0.42	0.38	0.4	1.7	1.5	1.6
60	0.41	0.48	0.4	1.6	1.9	1.8
61	0.48	0.54	0.5	1.9	2.2	2.0
62	0.54	0.6	0.6	2.2	2.4	2.3
63	0.45	0.52	0.5	1.8	2.1	1.9
64	0.3	0.32	0.3	1.2	1.3	1.2
65	0.43	0.2	0.3	1.7	0.8	1.3
66	0.61	0.54	0.6	2.4	2.2	2.3
67	0.6	0.57	0.6	2.4	2.3	2.3
68	0.59	0.62	0.6	2.4	2.5	2.4
69	0.62	0.69	0.7	2.5	2.8	2.6
70	0.68	0.7	0.7	2.7	2.8	2.8
71	0.58	0.47	0.5	2.3	1.9	2.1
72	0.42	0.43	0.4	1.7	1.7	1.7
73	0.63	0.65	0.6	2.5	2.6	2.6
74	0.73	0.75	0.7	2.9	3.0	3.0
75	0.66	0.44	0.6	2.6	1.8	2.2
76	0.55	0.59	0.6	2.2	2.4	2.3
77	0.45	0.49	0.5	1.8	2.0	1.9
78	0.5	0.54	0.5	2.0	2.2	2.1
79	0.53	0.59	0.6	2.1	2.4	2.2
80	0.6	0.49	0.5	2.4	2.0	2.2
81	1	0.59	0.8	4.0	2.4	3.2

Plant number	**Weight of damaged grains/ sample (R-1)**	**Weight of damaged grains/ sample (R-2)**	**Mean weight in grams**	**Percent damage intensity R1**	**Percent damage intensity R2**	**Mean damage intensity (%)**
82	0.53	0.55	0.5	2.1	2.2	2.2
83	0.91	0.53	0.7	3.6	2.1	2.9
84	0.58	0.49	0.5	2.3	2.0	2.1
85	0.37	0.5	0.4	1.5	2.0	1.7
86	0.61	0.74	0.7	2.4	3.0	2.7
87	0.61	0.73	0.7	2.4	2.9	2.7
88	0.62	0.75	0.7	2.5	3.0	2.7
89	0.63	0.51	0.6	2.5	2.0	2.3
90	0.65	0.49	0.6	2.6	2.0	2.3
91	0.66	0.95	0.8	2.6	3.8	3.2
92	0.36	0.44	0.4	1.4	1.8	1.6
93	0.55	0.57	0.6	2.2	2.3	2.2
94	0.49	0.73	0.6	2.0	2.9	2.4
95	0.5	0.55	0.5	2.0	2.2	2.1
96	0.5	0.53	0.5	2.0	2.1	2.1
97	0.27	0.31	0.3	1.1	1.2	1.2
98	0.64	0.66	0.7	2.6	2.6	2.6
99	0.45	0.47	0.5	1.8	1.9	1.8
100	1.2	1.23	1.2	4.8	4.9	4.9
101	0.43	0.66	0.5	1.7	2.6	2.2
102	0.31	0.45	0.4	1.2	1.8	1.5
103	0.37	0.48	0.4	1.5	1.9	1.7
104	0.26	0.38	0.3	1.0	1.5	1.3
105	0.28	0.3	0.3	1.1	1.2	1.2
106	0.29	0.31	0.3	1.2	1.2	1.2
107	0.28	0.31	0.3	1.1	1.2	1.2
108	0.27	0.3	0.3	1.1	1.2	1.1
109	0.45	0.54	0.5	1.8	2.2	2.0

Plant number	**Weight of damaged grains/ sample (R-1)**	**Weight of damaged grains/ sample (R-2)**	**Mean weight in grams**	**Percent damage intensity R1**	**Percent damage intensity R2**	**Mean damage intensity (%)**
110	0.54	0.57	0.6	2.2	2.3	2.2
111	0.56	0.55	0.6	2.2	2.2	2.2
112	0.58	0.51	0.5	2.3	2.0	2.2
113	0.24	0.57	0.4	1.0	2.3	1.6
114	0.49	0.51	0.5	2.0	2.0	2.0
115	0.48	0.41	0.4	1.9	1.6	1.8
116	0.55	0.57	0.6	2.2	2.3	2.2
117	0.5	0.37	0.4	2.0	1.5	1.7
118	0.27	0.31	0.3	1.1	1.2	1.2
119	0.73	0.52	0.6	2.9	2.1	2.5
120	1	0.89	0.9	4.0	3.6	3.8
121	0.75	0.72	0.7	3.0	2.9	2.9
122	0.35	0.32	0.3	1.4	1.3	1.3
123	0.43	0.27	0.4	1.7	1.1	1.4
124	0.44	0.5	0.5	1.8	2.0	1.9
125	0.45	0.61	0.5	1.8	2.4	2.1
126	0.59	0.62	0.6	2.4	2.5	2.4
127	0.6	0.62	0.6	2.4	2.5	2.4
128	0.62	0.64	0.6	2.5	2.6	2.5
129	0.6	0.65	0.6	2.4	2.6	2.5
130	0.6	0.66	0.6	2.4	2.6	2.5
131	0.4	0.35	0.4	1.6	1.4	1.5
132	0.48	0.52	0.5	1.9	2.1	2.0
133	0.34	0.68	0.5	1.4	2.7	2.0
134	0.63	0.72	0.7	2.5	2.9	2.7
135	0.54	0.7	0.6	2.2	2.8	2.5
136	0.46	0.69	0.6	1.8	2.8	2.3
137	0.43	0.45	0.4	1.7	1.8	1.8

Plant number	Weight of damaged grains/ sample (R-1)	Weight of damaged grains/ sample (R-2)	Mean weight in grams	Percent damage intensity R1	Percent damage intensity R2	Mean damage intensity (%)
138	0.39	0.37	0.4	1.6	1.5	1.5
139	1.34	0.83	1.1	5.4	3.3	4.3
140	0.41	0.51	0.5	1.6	2.0	1.8
141	0.43	0.52	0.5	1.7	2.1	1.9
142	0.5	0.55	0.5	2.0	2.2	2.1
143	0.53	0.57	0.6	2.1	2.3	2.2
144	0.48	0.52	0.5	1.9	2.1	2.0
145	0.32	0.37	0.3	1.3	1.5	1.4
146	0.66	0.64	0.7	2.6	2.6	2.6
147	0.55	0.11	0.3	2.2	0.4	1.3
148	0.6	0.57	0.6	2.4	2.3	2.3
149	0.65	0.6	0.6	2.6	2.4	2.5
150	0.72	0.6	0.7	2.9	2.4	2.6
151	0.4	0.27	0.3	1.6	1.1	1.3
152	0.57	0.53	0.6	2.3	2.1	2.2
153	0.31	0.31	0.3	1.2	1.2	1.2
154	0.36	0.39	0.4	1.4	1.6	1.5
155	0.4	0.57	0.5	1.6	2.3	1.9
156	0.84	0.33	0.6	3.4	1.3	2.3
157	0.28	0.48	0.4	1.1	1.9	1.5
158	0.63	0.48	0.6	2.5	1.9	2.2
159	0.37	0.74	0.6	1.5	3.0	2.2
160	0.3	0.56	0.4	1.2	2.2	1.7
161	0.52	0.4	0.5	2.1	1.6	1.8
162	0.45	0.4	0.4	1.8	1.6	1.7
163	0.4	0.4	0.4	1.6	1.6	1.6
164	0.15	0.36	0.3	0.6	1.4	1.0
165	0.3	0.73	0.5	1.2	2.9	2.1

Plant number	**Weight of damaged grains/ sample (R-1)**	**Weight of damaged grains/ sample (R-2)**	**Mean weight in grams**	**Percent damage intensity R1**	**Percent damage intensity R2**	**Mean damage intensity (%)**
166	0.6	0.51	0.6	2.4	2.0	2.2
167	0.4	0.31	0.4	1.6	1.2	1.4
168	0.11	0.14	0.1	0.4	0.6	0.5
169	0.32	0.42	0.4	1.3	1.7	1.5
170	0.13	0.34	0.2	0.5	1.4	0.9
171	0.17	0.21	0.2	0.7	0.8	0.8
172	0.23	0.31	0.3	0.9	1.2	1.1
173	0.32	0.43	0.4	1.3	1.7	1.5
174	0.36	0.49	0.4	1.4	2.0	1.7
175	0.34	0.47	0.4	1.4	1.9	1.6
176	0.66	0.26	0.5	2.6	1.0	1.8
177	0.29	0.36	0.3	1.2	1.4	1.3
178	0.32	0.34	0.3	1.3	1.4	1.3
179	0.35	0.39	0.4	1.4	1.6	1.5
180	0.34	0.37	0.4	1.4	1.5	1.4
181	0.41	0.46	0.4	1.6	1.8	1.7
182	0.39	0.42	0.4	1.6	1.7	1.6
183	0.37	0.36	0.4	1.5	1.4	1.5
184	0.43	0.49	0.5	1.7	2.0	1.8
185	0.62	0.64	0.6	2.5	2.6	2.5
186	0.4	0.45	0.4	1.6	1.8	1.7
187	0.41	0.46	0.4	1.6	1.8	1.7
188	0.35	0.41	0.4	1.4	1.6	1.5
189	0.34	0.26	0.3	1.4	1.0	1.2
190	0.4	0.48	0.4	1.6	1.9	1.8
191	0.92	0.85	0.9	3.7	3.4	3.5
192	0.44	0.47	0.5	1.8	1.9	1.8
193	0.42	0.47	0.4	1.7	1.9	1.8

Plant number	Weight of damaged grains/ sample (R-1)	Weight of damaged grains/ sample (R-2)	Mean weight in grams	Percent damage intensity R1	Percent damage intensity R2	Mean damage intensity (%)
194	0.6	0.49	0.5	2.4	2.0	2.2
195	0.5	0.59	0.5	2.0	2.4	2.2
196	0.66	0.47	0.6	2.6	1.9	2.3
197	0.3	0.34	0.3	1.2	1.4	1.3
198	0.45	0.08	0.3	1.8	0.3	1.1
199	0.4	0.38	0.4	1.6	1.5	1.6
200	0.45	0.48	0.5	1.8	1.9	1.9
201	0.36	0.39	0.4	1.4	1.6	1.5
202	0.4	0.45	0.4	1.6	1.8	1.7
203	0.45	0.47	0.5	1.8	1.9	1.8
204	0.18	0.47	0.3	0.7	1.9	1.3
205	0.55	0.82	0.7	2.2	3.3	2.7
206	0.4	0.34	0.4	1.6	1.4	1.5
207	0.45	0.47	0.5	1.8	1.9	1.8
208	0.47	0.48	0.5	1.9	1.9	1.9
209	0.4	0.3	0.4	1.6	1.2	1.4
210	0.35	0.4	0.4	1.4	1.6	1.5
211	0.18	0.24	0.2	0.7	1.0	0.8
212	0.35	0.83	0.6	1.4	3.3	2.4
213	0.42	0.51	0.5	1.7	2.0	1.9
214	0.31	0.5	0.4	1.2	2.0	1.6
215	0.27	0.51	0.4	1.1	2.0	1.6
216	0.22	0.58	0.4	0.9	2.3	1.6
217	0.23	0.55	0.4	0.9	2.2	1.6
218	0.24	0.5	0.4	1.0	2.0	1.5
219	0.27	0.27	0.3	1.1	1.1	1.1
220	0.26	0.24	0.3	1.0	1.0	1.0
221	0.65	0.34	0.5	2.6	1.4	2.0

Plant number	**Weight of damaged grains/ sample (R-1)**	**Weight of damaged grains/ sample (R-2)**	**Mean weight in grams**	**Percent damage intensity R1**	**Percent damage intensity R2**	**Mean damage intensity (%)**
222	0.41	0.3	0.4	1.6	1.2	1.4
223	0.2	0.35	0.3	0.8	1.4	1.1
224	0.68	0.65	0.7	2.7	2.6	2.7
225	0.68	0.75	0.7	2.7	3.0	2.9
226	0.97	0.67	0.8	3.9	2.7	3.3
227	0.32	0.63	0.5	1.3	2.5	1.9
228	0.69	0.3	0.5	2.8	1.2	2.0
229	0.46	0.62	0.5	1.8	2.5	2.2
230	0.77	0.48	0.6	3.1	1.9	2.5
231	0.46	0.91	0.7	1.8	3.6	2.7
232	0.46	0.92	0.7	1.8	3.7	2.8
233	0.46	0.94	0.7	1.8	3.8	2.8
234	0.33	0.23	0.3	1.3	0.9	1.1
235	0.43	0.53	0.5	1.7	2.1	1.9
236	0.37	0.47	0.4	1.5	1.9	1.7
237	0.25	0.3	0.3	1.0	1.2	1.1
238	0.69	0.61	0.7	2.8	2.4	2.6
239	0.49	0.38	0.4	2.0	1.5	1.7
240	0.56	0.65	0.6	2.2	2.6	2.4
241	0.41	0.52	0.5	1.6	2.1	1.9
242	0.56	0.68	0.6	2.2	2.7	2.5
243	0.76	0.33	0.5	3.0	1.3	2.2
244	0.56	0.4	0.5	2.2	1.6	1.9
245	0.27	0.68	0.5	1.1	2.7	1.9
246	0.5	0.62	0.6	2.0	2.5	2.2
247	1.18	0.63	0.9	4.7	2.5	3.6
248	0.92	0.57	0.7	3.7	2.3	3.0
249	0.41	0.57	0.5	1.6	2.3	2.0

Plant number	Weight of damaged grains/ sample (R-1)	Weight of damaged grains/ sample (R-2)	Mean weight in grams	Percent damage intensity R1	Percent damage intensity R2	Mean damage intensity (%)
250	0.78	0.65	0.7	3.1	2.6	2.9
251	0.57	0.54	0.6	2.3	2.2	2.2
252	0.36	0.51	0.4	1.4	2.0	1.7
253	0.48	0.46	0.5	1.9	1.8	1.9
254	0.52	0.37	0.4	2.1	1.5	1.8
255	0.3	0.41	0.4	1.2	1.6	1.4

Table 4.2.1d: Mean and range of black pinhead damage (in grams) in cross PR121 X PR126:

Variable	**N**	**Mean ± SE**	**Range**
Replication 1	255	0.4695 ± 0.0118	0.11 – 1.34
Replication 2	255	0.4912 ± 0.0107	0.08 – 1.23
Mean	255	0.4803 ± 0.0099	0.11 – 1.21

The mean and range of number of grains with black pinhead damage was recorded for both the replications. For the cross PR121 X PR126 the average mean was recorded to be 0.4803 with range of 0.11 – 1.21.

The bell shaped curve on the graph represents the normal distribution of the black pinhead in cross PR121 X PR126 (Figure 4.2.1b).

The weight in grams per plant and percent damage intensity of black pinhead was observed for the parent variety PR125. A total of 20 plants were selected at random, threshed and milled. Variation was observed in damage intensity in individual plants with range of 1.28-4.44 (Table 4.2.1(e)). The black pinpoint damage in PAU 201 showed a damage intensity in the range of 0.47-2.53 per cent (Siruguri *et al* 2012).

Scoring of observed data based on percentage damage intensity:

Based on the observations recorded above scoring of data was done to understand the complexity of distribution of black pinhead damage in both the crosses. The number of plants under each score was counted and mean intensity of black pinhead under each score was established. Malakar *et al* (2009) did grading of discoloration on a scale of 0-5. The observations based on scoring were observed as follows:

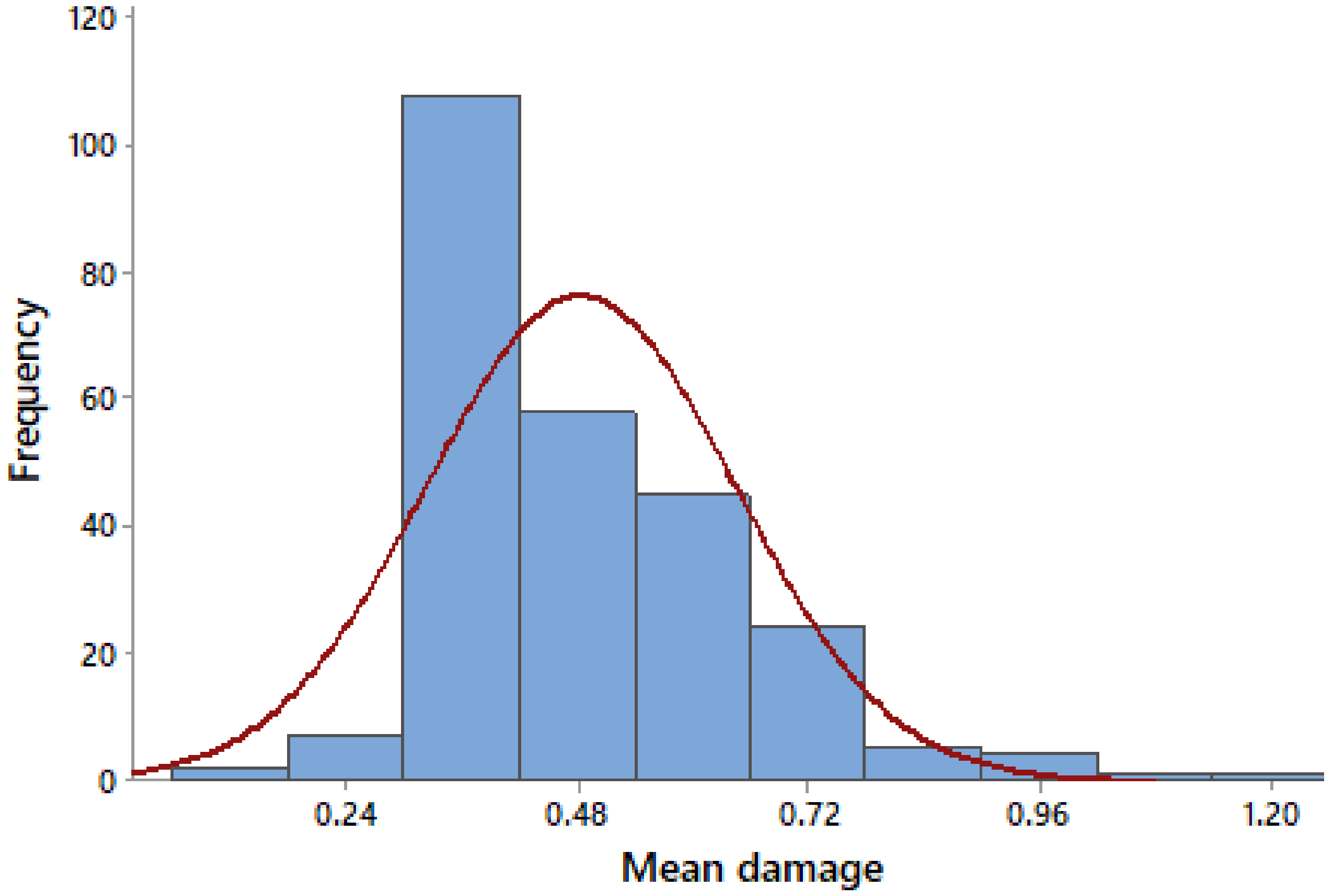

Figure 4.2.1b: Normal distribution of the black pinhead damage in cross PR121 X PR126.

Table 4.2.1 (e): Weight (grams) and per cent damage intensity of damaged grains per plant of PR 125

S. No.	Weight in grams	Per cent damage intensity
1	0.32	1.28
2	0.49	1.96
3	0.76	3.04
4	1.11	4.44
5	0.99	3.96
6	1.03	4.12
7	0.39	1.56
8	0.98	3.92
9	0.39	1.56
10	0.44	1.76
11	0.64	2.56
12	0.76	3.04
13	0.68	2.72
14	0.45	1.8
15	0.49	1.96
16	0.34	1.36
17	0.65	2.6
18	0.47	1.88
19	0.32	1.6
20	0.49	2.4

Table 4.2.1f: Scoring of data based on percent damage intensity, frequency of damage and percent frequency for the population of cross (PR121 X PR125)

Range	Score	No. of grains with black pinhead in PR125	No. of grains with black pinhead in F_3 seeds		Intensity of black pinhead in F_3 seeds		Mean intensity of black pinhead in F_3 grains
			R1	R2	R1	R2	
<1.0	1	0	26	18	10.1	7.0	13
1.0-2.0	2	10	104	118	40.7	46.1	129
2.0-3.0	3	4	80	92	31.2	35.9	77
3.0-4.0	4	4	27	14	10.5	5.5	22
>4.0	5	2	19	14	7.4	5.5	13

The number of plants under each score was counted based on their percent damage intensity. The highest number of plants was reported to fall under score 2 which represent a class of 1-2% damage. This indicates that maximum of number of plants had damage ranging from 1-2%. An average of 129 plants out of total 256 plants had percentage damage intensity ranging between 1-2%. The maximum number of plants for PR125 also come under score 2. In PR125 no plant was found with damage less than 1% as opposed to the crossed population which had about 7-10% plants with damage <1% (Table 4.2.1f)

The mean and range of black pinhead was calculated based on the scoring of data. The mean was found to be 50.8 with a range of 13–129.

Figure 4.2.1c shows the graphical representation of number of plants to score of damage intensity. Maximum number of plants had percentage damage ranging from 1-2%.

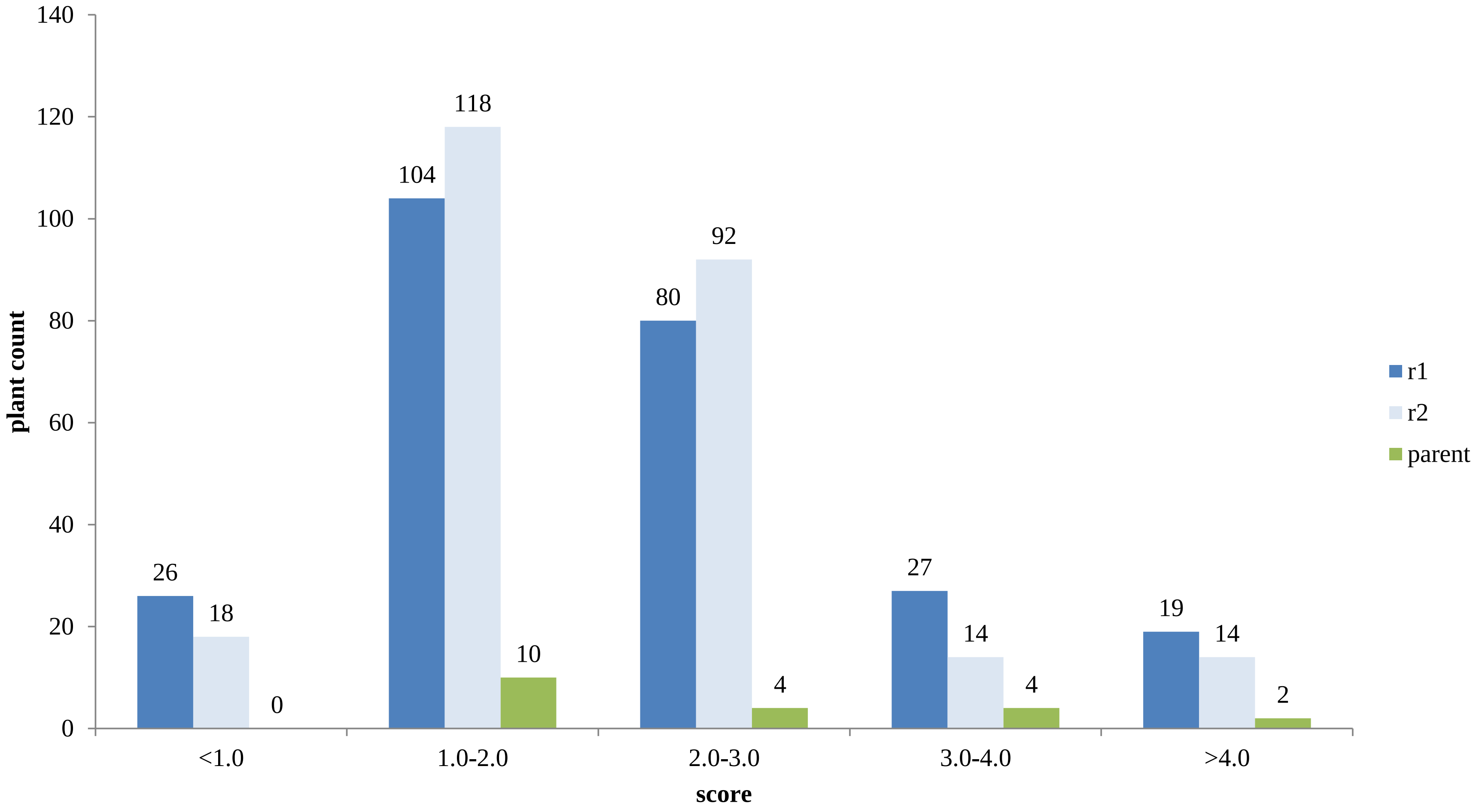

Figure 4.2.1c: **Graphical representation of no. of plants scored for damage in PR 125 and F_3 population derived from cross PR121 X PR125.**

Table 4.2.1g: Scoring of data based on percent damage intensity and number of grains with black pinhead for the population of cross (PR121 X PR126)

Range	Score	No. of grains with black pinhead in F_3 seeds		Intensity of black pinhead in F_3 seeds		Mean intensity of black pinhead in F_3 grains
		R1	R2	R1	R2	
<1.0	1	15	11	5.9	4.3	8
1.0-2.0	2	139	119	54.5	46.7	135
2.0-3.0	3	83	105	32.5	41.1	98
3.0-4.0	4	13	19	5.1	7.4	12
>4.0	5	5	1	2.0	0.5	2

The highest numbers of plants were reported to fall under score 2 which represent a class of 1-2% damage. This indicates that maximum number of plants had damage ranging from 1-2%. An average of 135 plants out of total 255 plants had percent damage intensity ranging between 1-2%. Only 8 plants had black pinhead damage intensity less than 1% and only 2 plants were found to have the highest percent intensity (>4%) of black pinhead (Table 4.2.1g).

Figure 4.2.1d shows the graphical representation of number of plants to score of damage intensity. Maximum number of plants had percentage damage ranging from 1-2%.

Highly significant variation was observed in F_3 progenies of both the crosses for black pinhead damage (Table 4.2.1h, 4.2.1i). However there were differences in the broad sense heritability (h^2) in two crosses. The broad sense heritability in cross PR121 X PR125 was 0.7, while it was 0.3 in cross PR121 X PR126.

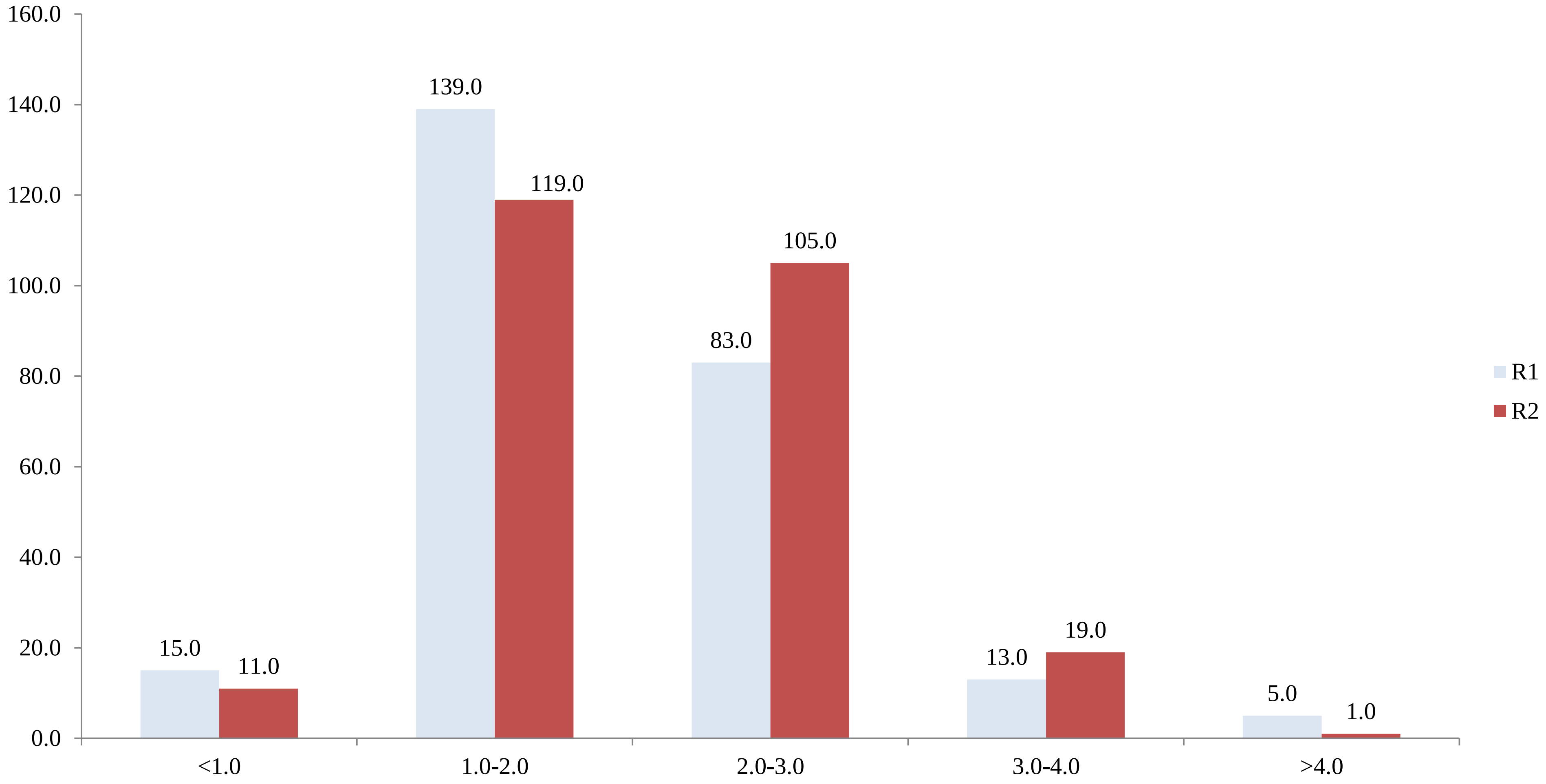

Figure 4.2.1d: Graphical representation of no. of plants scored for damage in the F_3 population derived from cross PR121 X PR126.

Table 4.2.1h: ANOVA for black pinhead damage in cross PR121 X PR125

Source of variation	Degree of Freedom	Sum of Squares	Mean sum of squares	F value	Pr>F
Replication	1	0.42	0.42	0.88	0.348
Genotype	255	475.8	1.86	3.95	<0.0001
Error	255	120.4	0.47	-	-
Total	511	596.7	1.17	-	-

Standard error mean (SEM) = √MSE/R= √0.235= 0.48

$$\text{Broad sense heritability } (\sigma^2 g) = \frac{\text{MSG} - \text{MSE}}{\text{R}} = 0.7$$

Table 4.2.1i: ANOVA for black pinhead damage in cross PR121 X PR126

Source of variation	Degree of Freedom	Sum of Squares	Mean sum of squares	F value	Pr>F
Replication	1	0.96	0.96	4.36	0.039
Genotype	254	205.3	0.81	3.58	<0.0001
Error	254	57.3	0.22	-	-
Total	509	263.6	0.52	-	-

Standard error mean (SEM) = √MSE/R= 0.3

$$\text{Broad sense heritability } (\sigma^2 g) = \frac{\text{MSG} - \text{MSE}}{\text{R}} = 0.3$$

The difference in the heritability could be due to difference in the genetic constitution for black pinhead damage in PR125 and PR126. The PR125 has high black pinhead damage as compared to PR126 (Table 4.2.1h and table 4.2.1i).

4.2.2 (a) : Inheritance of black pinhead

Distribution analysis was done and chi square test was conducted to study the inheritance of trait. The data was found to be distributed normally showing the quantitative nature of the trait (Figure 4.2.2a and 4.2.2b).

Figure 4.2.2a: Distribution of black pinhead damage in F_3 grains of cross PR121 X PR125. Since p-value is >0.05 so we accept the null hypothesis. Hence the data is normally distributed.

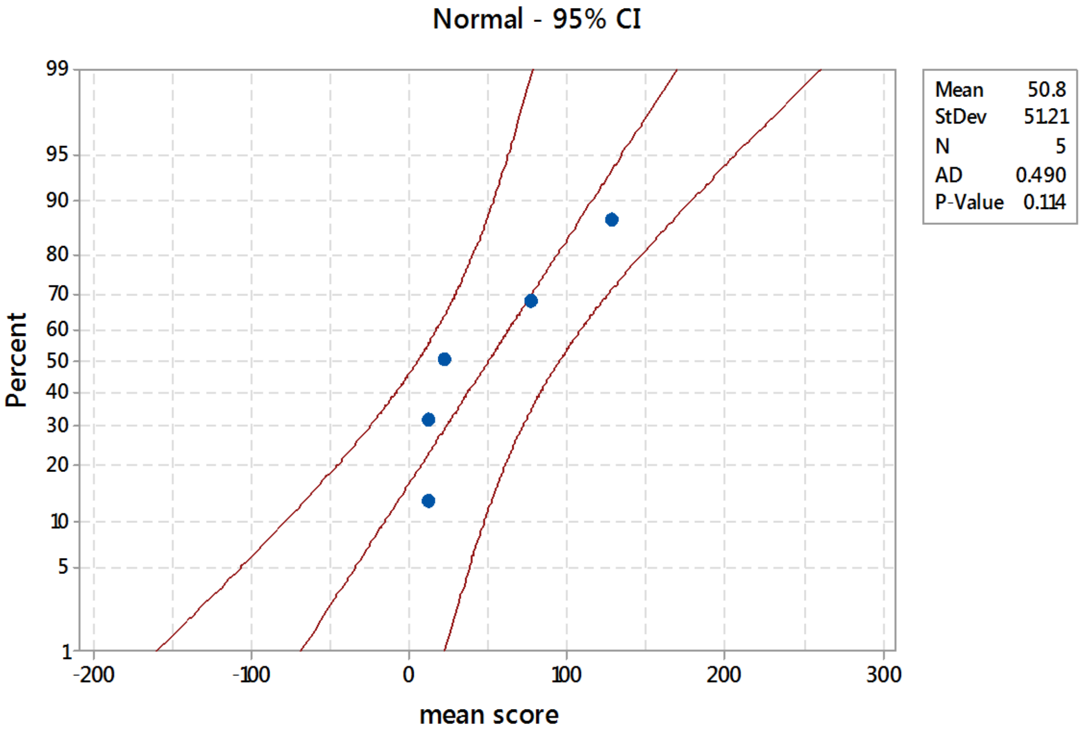

Figure 4.2.2 a: Inheritance of black pinhead in cross PR121 X PR125.

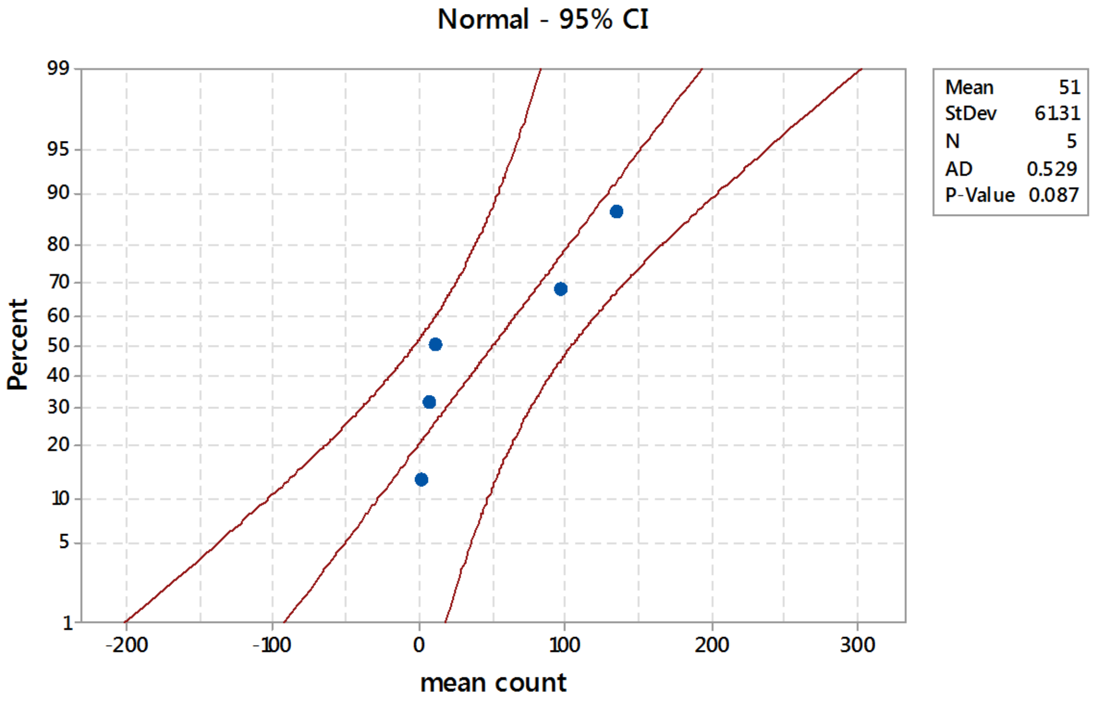

Figure 4.2.2 b: Inheritance of black pinhead in cross PR121 X PR126.

Figure 4.2.2b: Distribution of black pinhead damage in F_3 grains of cross PR121 X PR126. Since p-value is >0.05 so we accept the null hypothesis. Hence the data is normally distributed.

Although the black pinhead was found to be normally distributed, chi square analysis was carried out to calculate the number of genes involved in controlling this character. Considering plants with < 1% damage to be undamaged and all other plants damaged, the ratio of damaged to undamaged is 243: 18 = 18: 1 which is approximately equal to 15: 1 ratio of duplicate gene action controlled by two genes.

Chi square inheritance for cross PR121 X PR125

Null Hypothesis Ho: Data fits in 15:1 ratio

Range of percentage damage intensity	Observed	Expected
<1%	13	16
1 - >4%	243	246

$$\chi^2 = \frac{(O - E)^2}{E} \times 100$$

$$\chi^2 = \frac{(13 - 16)^2}{16} + \frac{(243 - 240)^2}{16} = 0.56 + 0.04 = 0.6$$

Since p-value is 0.439 > 0.05, so results are significant. Thus the data fits in 15:1 ratio and shows duplicate gene action controlled by 2 genes.

Chi square inheritance for cross PR121 X PR126

Range of percentage damage intensity	Observed	Expected
<1%	8	16
1 - >4%	247	239

$$\chi^2 = \frac{(O - E)^2}{E} \times 100$$

$$\chi^2 = \frac{(8 - 16)^2}{16} + \frac{(247 - 239)^2}{16} = 4.0 + 0.27 = 4.27$$

Since p- value 0.040<0.05, so results are not significant. This represents that the character in this case was not controlled by 2 genes as opposed to the previous cross. So the control of black pinhead in the cross PR121 X PR126 can be attributed to QTLs (Cochran and Cox 1992).

The differences in the inheritance of black pinhead damage in two different crosses can be due to differential level of black pinhead damage in parental lines PR125 and PR126. The PR125 shows higher damage as compared to PR126. It might be possible that trait in PR125 is being controlled by interaction of two genes with several minor effect QTLs.

4.3 Experiment III: Bulked segregant analysis to find out association of rice SSR markers with damage in rice grains.

Bulked segregant analysis was performed to evaluate the markers which were polymorphic for the character in the parents as well as in populations. SSR markers were used for conducting parental polymorphism and the polymorphic primers were used to investigate polymorphism among the bulks. The list of markers used in this study are given in the table below:

Table 4.3.1 List of Markers applied

Marker	Chromosome no.	Marker	Chromosome no.
RM 6464 (M)	1	RM 15065 (M)	3
RM 6470 (M)	1	RM 7642 (M)	3
RM 1329 (M)	1	RM 3646 (M)	3
RM 600 (M)	1	RM 15567 (M)	3
RM 594 (M)	1	RM 3525 (M)	3
RM 8004 (M)	1	RM 422 (M)	3
RM 1196 (M)	1	RM 16147 (M)	3
RM 5 (M)	1	RM 85 (M)	3
RM 7341 (M)	1	RM 551 (P)	4
RM 315 (M)	1	RM 16493 (P)	4
RM 3362 (M)	1	RM 16559 (P)	4
RM6842 (M)	2	RM 16720 (P)	4
RM 6367 (M)	2	RM 142 (P)	4
RM 12478 (M)	2	RM 1018 (P)	4

Marker	Chromosome no.	Marker	Chromosome no.
RM 12923 (M)	2	RM 349 (P)	4
RM 550 (M)	2	RM 507 (M)	5
RM 13170 (M)	2	RM 17863 (M)	5
RM 6617 (M)	2	RM 17959 (M)	5
RM 221 (M)	2	RM 169 (M)	5
RM 341 (M)	2	RM 7588 (M)	5
RM 5807 (M)	2	RM 18439 (M)	5
RM 318 (M)	2	RM 3486 (M)	5
RM 425 (M)	2	RM 7423 (M)	5
RM 208 (M)	2	RM 5907 (M)	5
RM 7337 (M)	2	RM 6775 (M)	6
RM 3654 (M)	3	RM 510 (M)	6
RM 132 (M)	3	RM 19620 (M)	6
RM 22 (M)	3	RM 5963 (M)	6
RM 5474 (M)	3	RM 5745 (M)	6
RM 14898 (M)	3	RM 1161 (M)	6
RM 103 (M)	6	RM 25149 (M)	10
RM 295 (M)	7	RM 239 (M)	10
RM 21242 (M)	7	RM 25366 (M)	10
RM 21328 (P)	7	RM 184 (P)	10
RM 542 (P)	7	RM 25663 (M)	10
RM 5543 (P)	7	RM 147 (M)	10
RM 11 (M)	7	RM 3834 (M)	10
RM 455 (M)	7	RM 6673 (M)	10
RM 18 (M)	7	RM 6327 (M)	11
RM 22105 (P)	7	RM 26105 (M)	11
RM 248 (P)	7	RM 116 (P)	11
RM 408 (P)	8	RM 536 (P)	11
RM 3702 (M)	8	RM 5590 (M)	11

Marker	**Chromosome no.**	**Marker**	**Chromosome no.**
RM 547 (M)	8	RM 26643 (P)	11
RM 8243 (M)	8	RM 209 (M)	11
RM 6471 (M)	8	RM 206 (M)	11
RM 5999 (M)	8	RM 27154 (M)	11
RM 22997 (M)	8	RM 27318 (M)	11
RM 23174 (M)	8	RM 5568 (M)	12
RM 210 (P)	8	RM 7315 (P)	12
RM 3452 (M)	8	RM 27542 (P)	12
RM 6948 (M)	8	RM 27706 (P)	12
RM 264 (M)	8	RM 1036 (M)	12
RM 23661 (M)	9	RM 27962 (M)	12
RM 5799 (P)	**9**	RM 28118 (M)	12
RM 296 (P)	9	RM 6022 (M)	12
RM 409 (P)	9	RM 28404 (M)	12
RM 257 (P)	9	RM 28607 (M)	12
RM 6491 (P)	9	RM 1159 (P)	12
RM 24740 (P)	9	RM 6306 (P)	12
RM 5095 (M)	10		
RM 474 (M)	10		
RM 6404 (M)	10		

Approximately 200 SSR markers were used to conduct parental polymorphism, out of which 30 markers were found to be polymorphic between the two parents. A total of 15% polymorphism was detected between the two parents viz., PR121 and PR125 (Plate 7). No markers were found to be polymorphic for chromosome number 1, 2, 3, 5 and 6. Polymorphism was detected to be 50% for chromosome number 7. Approximately 16% polymorphism was seen for chromosome 8. Chromosome 9 and 10 had 85% and 9% polymorphism, respectively. Chromosome 11 and 12 showed 30% and 41% polymorphism, respectively. Hundred per cent polymorphism was recorded for chromosome number 4 (Table 4.3.2).

The polymorphic chromosome specific markers were then applied to the bulks. Orjuela *et al* (2010) developed a universal core genetic map for rice using SSR markers. Two bulks were prepared one with least or no damage and the other having plants with maximum damage. These plants were selected based on the data collected by taking weight of damaged grains.

SSR primer pairs which generated polymorphism between PR121 and PR125 were used to detect the putative markers associated with specific phenotype. The bulk containing DNA from plants with least damage or no damage had the bands similar to those of PR121 and the ones with maximum damage had bands similar to PR125. Markers RM 551, RM 7315 and RM 1159 were also found to be polymorphic in the bulks (Plate 8). Salunkhe *et al* 2011 also used SSR makers to find out polymorphism between the parental lines and then the markers found polymorphic between the parents were applied to resistant and susceptible bulks using bulked segregant analysis.

Table 4.3.2: Percentage polymorphism for each chromosome.

Chromosome No.	No. of Markers Applied	Polymorphic	Percentage Polymorphism
1	11	-	-
2	14	-	-
3	13	-	-
4	07	07	100
5	09	-	-
6	10	-	-
7	10	05	50
8	12	02	16
9	7	06	85
10	11	01	9
11	10	03	30
12	12	05	41

The images for parental polymorphism i.e. polymorphism between the parents PR121 and PR125 was taken using the gel doc system. The agarose gel was loaded with the amplified product and run on the electrophoresis unit. The images for both the parental polymorphism and bulked segregant analysis (BSA) were recorded as shown in Plate 7 and 8. Plate 7 shows the images for parental polymorphism whereas plate 8 shows the image for bulked segregant analysis.

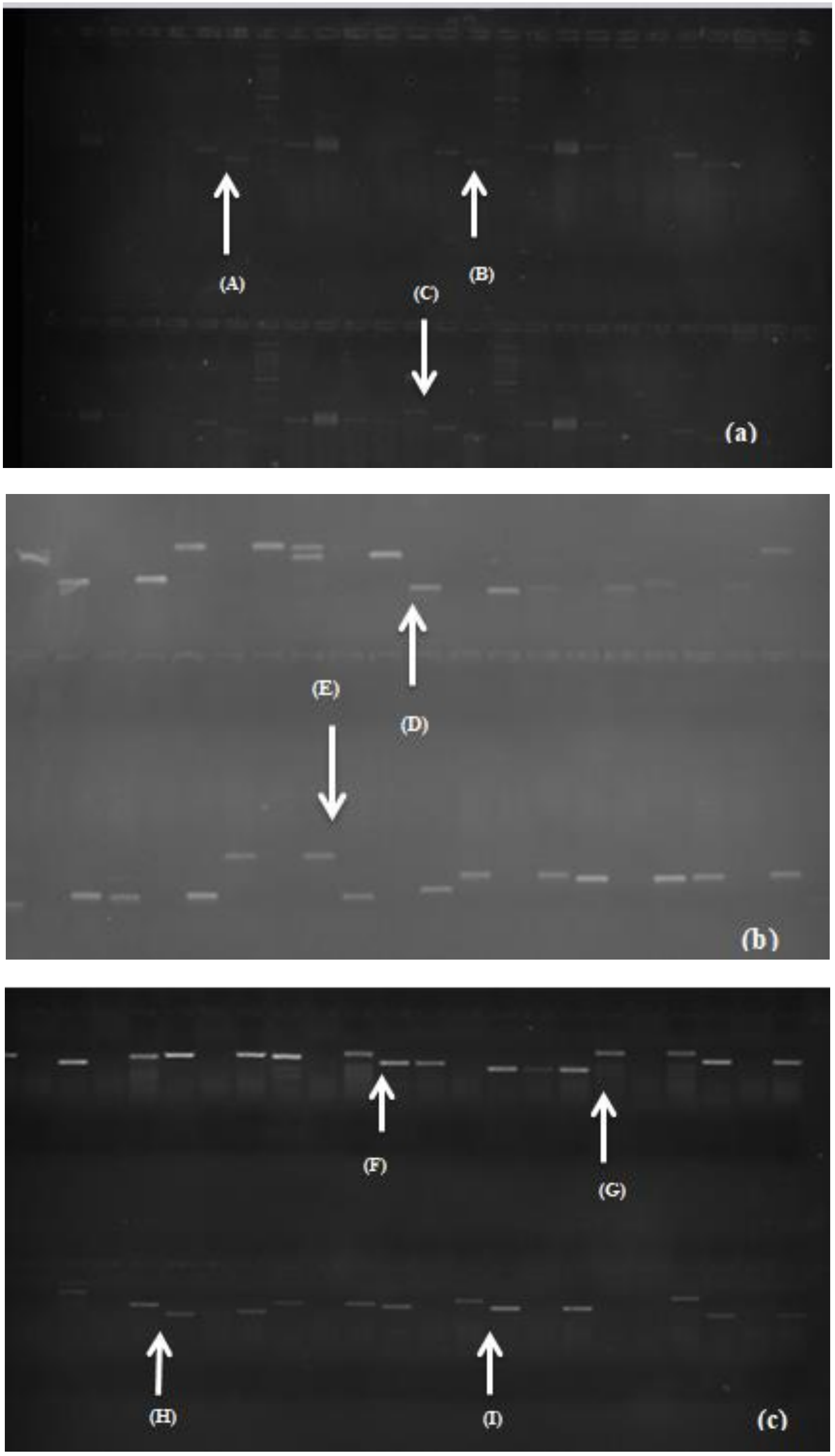

Plate 7: Parental polymorphism with respect to SSR markers. Figure (a, b and c): parental polymorphism between PR121 and PR125. Arrows marked represents the markers showing polymorphism. A: RM 551, B: RM 16493, C: RM 16720, D: RM 6471, E: RM 210, F: RM 116, G: RM 556, H: RM 26643, I: RM 7315.

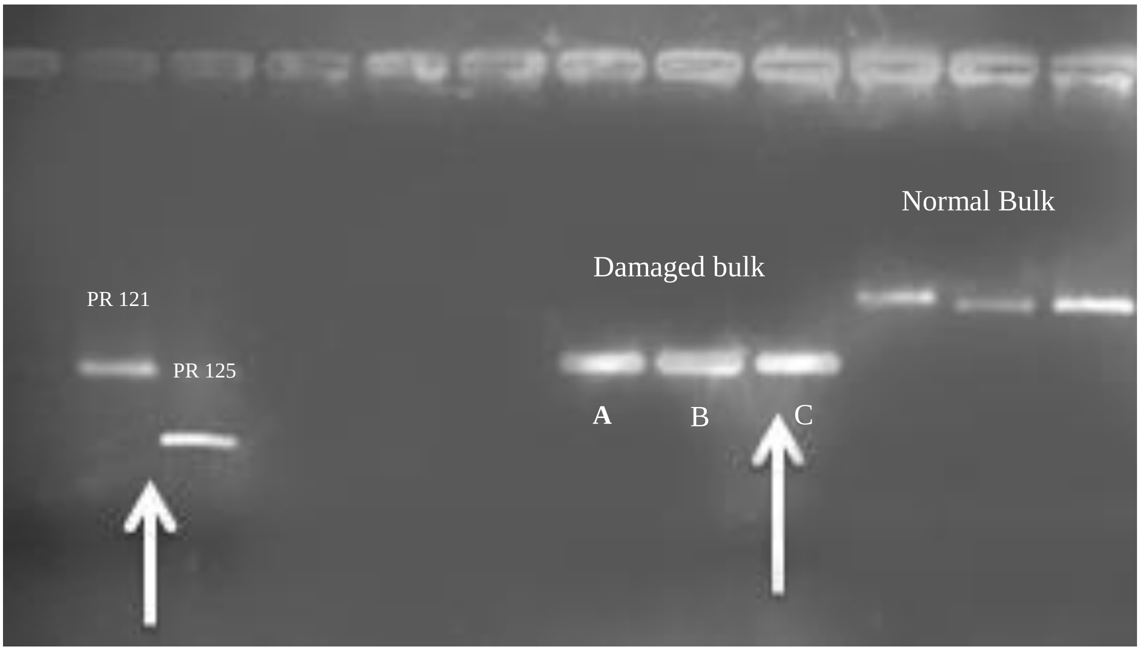

Plate 8: Polymorphism shown by markers: A: RM 551, B: RM 7315, C: RM 1159 in the parents as well as bulks. The damaged bulk shows bands similar to that of PR125 (damaged parent with pinhead damage) and normal bulk shows bands similar to that of PR121 (undamaged parent i.e. no black pinhead damage).

BSA is a rapid and shortcut method to identify chromosomal location of the target trait (Michelmore *et al* 1990). The 3 markers viz., RM 551, RM 7315 and RM 1159 showed expected pattern in the BSA (Plate 8). However these markers still need to be further investigated. Zhang *et al* (2009) also used bulked segregant analysis to detect QTL related to heat tolerance. They found two markers to be polymorphic between the bulks.

In the previous studies there were speculations of black pinhead damage due to bacterial or viral contamination. Even though these speculations were nullified in the previous studies, but those could not establish the genetic control of this trait. The present study not only nullified any damage due to contamination on seed, but also tried to prove that the trait has genetic control as it showed genetic segregation. The electron microscopy analysis of damaged grains showed damaged starch granules at the point of damage, though which is not very deep inside the grain, but localized on the upper layers of the grain. The markers identified in this study can be investigated further to identify genetic nature of the trait.

CHAPTER-V

SUMMARY

The studies entitled **"Anatomical and genetic analysis of damaged grains in rice (*Oryza sativa* L.)"** were conducted at Rice experimental area, Punjab Agricultural University, Ludhiana, India during *Kharif* 2017, to investigate the anatomical and genetic causes for damage in rice grains and to study the inheritance of the character. Experiment-I was conducted at Electron Microscopy Lab, PAU, Ludhiana. It was laid out to investigate the damage at cellular level. The imaging was done using Scanning Electron Microscope for the parents as well as the F_2 generation. Experiment-II was conducted at Rice experimental area, PAU to determine the extent of damage and percent damage intensity of plants. This experiment was conducted to understand the inheritance of the damage trait. Experiment- III was conducted at Central Molecular Biology Laboratory, Department of Plant Breeding and Genetics, PAU. This experiment was laid out to understand the genetic aspect of damage. SSR markers were used to conduct this experiment. The treatments were studied for cellular and genetic effect on damage. The results of investigations are summarized in this chapter as below:

The damaged and undamaged part of the same grain showed difference in the structure of starch granules. The starch granules in the normal or undamaged region were found to be compactly packed having normal granular structure with no kind of distortion whereas the damaged region of the same grain had difference in structure of starch granules. The starch granules in the damaged region were found to be loosely packed and had empty voids in between. The starch granules were basically broken.

There were no signs of fungal or any other kind of microbial contamination seen. The grains showed easy breakage during milling at the point of damage thus reducing the milling quality and milling percentage. The grains with no damage did not show easy breakage during milling. The cut sections through the point of damage represented that the pinpoint damage was not attributed to surface only but it was deeply embedded inside the grain but it did not cause any damage to the embryo.

The grains of both the populations of crosses PR121 × PR125 and PR121 × PR126 were milled and then sorted into damaged and normal grains. The weight of damaged grains per plant was recorded and percent damage intensity was calculated. Scoring was done for extent of damage on a scale of 1-5. Maximum number of plants had damage intensity ranging from 1-2% for both the populations and they were given the score 2. Similar trend was seen the parent PR125 for which 20 samples were weighed which also showed maximum number of plants under the score 2 i.e. 1-2% damage intensity.

The chi square analysis was carried out for both the populations to understand the complexity of this character. The trait was found to show the 15:1 ratio of inheritance for the cross PR121 × PR125. This states that the genes controlling the damage showed duplicate gene action and was found to be controlled by 2 genes. The data of the other cross PR121 × PR126 did not show any kind of Mendelian ratio of inheritance.

The analysis of variance (ANOVA) was carried out for both the populations the results of which represented that there was no significant difference between the replications for cross PR 121 × PR 125 but replications for cross PR 121 × PR126 were found to be significantly different. The treatments were found to be significantly different for both the crosses. The broad sense heritability (σ^2g) was reported to be higher in case of PR121 × PR125 i.e. 0.7 and lower in case of PR121 × PR126 i.e. 0.3.

The data for both the populations had normal distribution i.e. the data values were found to cluster around the mean. Though the chi square analysis suggested a 2 gene control in population of cross PR121 × PR125 yet the data showed normal distribution which suggested that there could be QTLs linked to the trait of interest with major 2 QTLs which could be linked to starch synthesizing genes.

The differences in the inheritance of black pinhead damage in two different crosses can be due to differential level of black pinhead damage in parental lines PR125 and PR126. The PR125 shows higher damage as compared to PR126. It might be possible that trait in PR125 is being controlled by interaction of two genes with several minor effect QTLs.

The molecular analysis using SSR markers revealed only 15% polymorphism between the parents. The polymorphic markers when applied to bulks showed segregation as parents. Only 3 markers showed polymorphism between the bulks. The bulk from normal or undamaged plant DNA showed bands relevant to PR121 and the bulk from damaged plant DNA showed bands relevant to PR125.

Hence, it may be concluded that the damage in rice grains can be attributed to starch malformation at cellular level and degradation of amylose and amylopectin in the starch granules which may further be controlled by 2 or more genes as suggested by chi square analysis of the percent damage intensity. The present study nullified any damage due to contamination on seed, and also tried to prove that the trait has genetic control as it showed genetic segregation. The electron microscopy analysis of damaged grains showed damaged starch granules at the point of damage. The markers identified in this study can be investigated further to identify genetic nature of the trait.

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VITA

Name of the student : Ritika Batra

Father's name : Mr. Prem Sagar Batra

Mother's name : Mrs. Seema Batra

Nationality : Indian

Date of Birth : 18.11.1994

Permanent Address : H.No. 209/1, Opp. Shining Printing Press
Prem Garh, Sutehri Road, Hoshiarpur

EDUCATIONAL QUALIFICATION

Bachelor degree : B.Sc. (Agriculture)

University and year of award : Punjab Agricultural University, Ludhiana
2016

OCPA : 8.04/10.00

Master's degree : M.Sc. (Plant Breeding and Genetics)

University and year of award : Punjab Agricultural University, Ludhiana
2019

OCPA : 8.14/10.00

Title of Master's Thesis : Anatomical and genetic analysis of damaged grains in rice (*Oryza sativa* L.)

Award/Distinction/
Fellowship/Scholarship : Merit Scholarship during M.Sc.