BIOLOGY OF *Ustilaginoidea virens* (Cooke.) Takahashi AND MAPPING QTL FOR RESISTANCE TO FALSE SMUT IN RICE (*Oryza sativa* L.)

Dissertation

Submitted to the Punjab Agricultural University
in partial fulfillment of the requirements
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DOCTOR OF PHILOSOPHY
in
PLANT PATHOLOGY
(Minor Subject: Biotechnology)

By

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(L-2017-A-48-D)

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LUDHIANA-141 004

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CERTIFICATE I

This is to certify that the dissertation entitled, **"BIOLOGY OF *Ustilaginoidea virens* (Cooke.) TAKAHASHI AND MAPPING QTL FOR RESISTANCE TO FALSE SMUT IN RICE (*Oryza sativa* L.)"** submitted for the degree of **Ph.D.** in the subject of **Plant Pathology** (Minor subject: **Biotechnology**) of the Punjab Agricultural University, Ludhiana, is a bonafide research work carried out by **Ishwinder Kamboj (**Admn. No. **L-2017-A-48-D)** under my supervision and that no part of this dissertation has been submitted for any other degree.

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

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CERTIFICATE II

This is to certify that the dissertation entitled, **"BIOLOGY OF *Ustilaginoidea virens* (Cooke.) TAKAHASHI AND MAPPING QTL FOR RESISTANCE TO FALSE SMUT IN RICE (*Oryza sativa* L.)"** submitted by **Ishwinder Kamboj (**Admn. No. **L-2017-A-48-D)** to the Punjab Agricultural University, Ludhiana, in partial fulfilment of the requirements for the degree of **Ph.D.,** in the subject of **Plant Pathology** (Minor subject: **Biotechnology**) has been approved by the Student's Advisory Committee after an oral examination on the same in collaboration with an External Examiner.

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ABSTRACT

The false smut of rice is caused by fungus *Ustilaginoidea virens* (*Villosciclava virens*). The disease causes 0.2-49% yield losses besides qualitative losses. The knowledge of disease cycle about the pathogen and the availability of resistant sources for false smut is lacking. The present study was conducted with various treatments for seed-borne, soil-borne and air-borne nature of primary inoculum in pot experiment. The disease incidence and percent infected panicles was more in case of plants with uncovered panicles (10-36%) than covered panicles (0-9%), in infected soil (9-33%) than in sterilized soil (8-13%) and in case of plants raised from infected seed (0-10%) than from healthy seed (0%). The percent infection was the highest in case of air-borne experiment in plants with uncovered panicles than in plants grown in infected soil whereas the plants raised from infected seeds reported only 5% infection. In another experiment, screening of 212 rice accessions against false smut disease under field conditions resulted in selection of 27 resistant lines. These lines were further screened under artificial conditions against the disease along with susceptible checks (GSR123 and PR116) resulted in selection of 19 resistant lines. Out of 19, the lines CANAROXA and IAC 47 with good agronomic trait were used as donors for preparing crosses with PR126 and PR116, respectively. Out of 78 SSR markers, the markers which show polymorphism in PR116 × IAC 47 includes RM16493, RM18457, RM25149 and RM28404 on chromosome 4, 5, 10 and 12, respectively. On the other hand, the polymorphic markers in case of PR126 × CANAROXA includes RM104, RM289, RM7434, RM8243, RM264, RM25149, RM10 and RM26643 on chromosome 1, 5, 6, 8, 10 and 11, respectively. These polymorphic markers were used to identify genetic diversity in the mapping population. However, the identified polymorphic markers in this study fail to depict the association between the marker and false smut resistance gene due to lack of definite pattern of markers at loci. Further analysis in next generation will help us to uncover the association between the marker and the false smut resistance gene.

Keywords: *Oryza sativa*, false smut, *Ustilaginoidea virens*, primary inoculum, resistance, QTLs

Signature of Major Advisor **Signature of the Student**

ਖੋਜ ਗ੍ਰੰਥ ਦਾ ਸਿਰਲੇਖ	:	*Ustilaginoidea virens* (Cooke.) Takahashi ਦਾ ਜੈਵਿਕ ਅਧਿਐਨ ਅਤੇ ਝੋਨੇ (*ਓਰਾਇਜ਼ਾ ਸਟੀਵਾ* ਐਲ.) ਵਿੱਚ ਝੂਠੀ ਕਾਂਗਿਆਰੀ ਰੋਗ ਦਾ ਟਾਕਰਾ ਕਰਨ ਵਾਲੇ QTL ਦੀ ਮੈਪਿੰਗ
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ਸਾਰ-ਅੰਸ਼

ਝੋਨੇ ਵਿੱਚ ਹੋਣ ਵਾਲਾ ਝੂਠੀ ਕਾਂਗਿਆਰੀ ਰੋਗ *Ustilaginoidea virens* (*Villosciclava virens*) ਉੱਲੀ ਕਾਰਨ ਹੁੰਦਾ ਹੈ। ਇਸ ਰੋਗ ਨਾਲ ਝਾੜ ਦਾ 0.2-49% ਤੱਕ ਨੁਕਸਾਨ ਤਾਂ ਹੁੰਦਾ ਹੀ ਹੈ ਪਰ ਇਸਦੇ ਨਾਲ ਹੀ ਨਾਲ ਝੋਨੇ ਦੀ ਗੁਣਵਤਾ ਵੀ ਘਟਦੀ ਹੈ। ਰੋਗ ਕਰਨ ਵਾਲੇ ਰੋਗਜਨਕ ਦੇ ਰੋਗ ਚੱਕਰ ਅਤੇ ਝੂਠੀ ਕਾਂਗਿਆਰੀ ਰੋਗ ਵਿਰੁੱਧ ਪ੍ਰਤੀਰੋਧਕਤਾ ਦੇਣ ਵਾਲੇ ਸਰੋਤਾਂ ਸਬੰਧੀ ਜਾਣਕਾਰੀ ਦੀ ਕਮੀ ਹੈ। ਮੌਜੂਦਾ ਅਧਿਐਨ ਦੌਰਾਨ ਪੋਟ ਤਜ਼ਰਬੇ ਵਿੱਚ ਬੀਜ, ਮਿੱਟੀ ਅਤੇ ਹਵਾ ਰਾਹੀਂ ਫੈਲਣ ਵਾਲੀ ਮੁੱਢਲੀ ਲਾਗ ਦੇ ਵੱਖੋ-ਵੱਖਰੇ ਉਪਚਾਰ ਕੀਤੇ ਗਏ। ਬੰਦ ਮੁੰਜ਼ਰਾਂ (0-9%) ਦੇ ਮੁਕਾਬਲੇ ਖੁਲ੍ਹੀਆਂ ਮੁੰਜ਼ਰਾਂ (10-36%), ਸਟਰਜ਼ਾਈਜ਼ਡ ਮਿੱਟੀ (8-13%) ਦੇ ਮੁਕਾਬਲੇ ਦੂਸ਼ਿਤ ਮਿੱਟੀ (9-33%) ਅਤੇ ਸਿਹਤਮੰਦ ਬੀਜ (0%) ਦੇ ਮੁਕਾਬਲੇ ਲਾਗ ਨਾਲ ਸੰਕ੍ਰਮਿਤ ਬੀਜ (0-10%) ਦੀ ਵਰਤੋਂ ਕਰਕੇ ਉਗਾਏ ਗਏ ਪੌਦਿਆਂ ਵਿੱਚ ਰੋਗ ਦੀ ਤੀਬਰਤਾ ਅਤੇ ਰੋਗੀ ਮੁੰਜਰਾਂ ਦੀ ਪ੍ਰਤੀਸ਼ਤਤਾ ਜ਼ਿਆਦਾ ਸੀ। ਲਾਗ ਦੀ ਪ੍ਰਤੀਸ਼ਤਤਾ ਹਵਾ ਜਨਿਤ ਤਜ਼ਰਬੇ ਵਿੱਚ ਦੂਸ਼ਿਤ ਮਿੱਟੀ ਵਿੱਚ ਉਗਾਏ ਗਏ ਪੌਦਿਆਂ ਦੇ ਮੁਕਾਬਲੇ ਖੁੱਲ੍ਹੀਆਂ ਮੁੰਜ਼ਰਾਂ ਵਾਲੇ ਪੌਦਿਆਂ ਵਿੱਚ ਲਾਗ ਦੀ ਪ੍ਰਤੀਸ਼ਤਤਾ ਜ਼ਿਆਦਾ ਸੀ ਜਦੋਂਕਿ ਉੱਲੀ ਯੁਕਤ ਬੀਜਾਂ ਦੀ ਵਤਰੋਂ ਕਰਕੇ ਉਗਾਏ ਗਏ ਪੌਦਿਆਂ ਵਿੱਚ ਰੋਗ ਦੀ ਪ੍ਰਤੀਸ਼ਤਤਾ ਸਿਰਫ 5% ਦਰਜ ਕੀਤੀ ਗਈ। ਇੱਕ ਹੋਰ ਤਜ਼ਰਬੇ ਵਿੱਚ, ਖੇਤ ਹਲਾਤਾਂ ਅਧੀਨ ਝੂਠੀ ਕਾਂਗਿਆਰੀ ਰੋਗ ਵਿਰੁੱਧ ਝੋਨੇ ਦੇ 212 ਅਕਸੈਸ਼ਨਾਂ ਦੀ ਜਾਂਚ ਕੀਤੀ ਗਈ ਅਤੇ ਇਹਨਾਂ ਵਿੱਚੋਂ ਇਸ ਰੋਗ ਦਾ ਟਾਕਰਾ ਕਰਨ ਦੇ ਸਮਰੱਥ 27 ਲਾਈਨਾਂ ਨੂੰ ਚੁਣਿਆ ਗਿਆ। ਇਹਨਾਂ 27 ਲਾਈਨਾਂ ਅਤੇ ਦੋ ਸੰਵੇਦਨਸ਼ੀਲ ਚੈੱਕ ਕਿਸਮਾਂ (ਜੀ ਐਸ ਆਰ ਅਤੇ ਪੀ ਆਰ 116) ਨੂੰ ਬਨਾਵਟੀ ਹਲਾਤਾਂ ਅਧੀਨ ਦੁਬਾਰਾ ਰੋਗ ਵਿਰੁੱਧ ਜਾਂਚਿਆ ਗਿਆ ਅਤੇ ਰੋਗ ਦਾ ਟਾਕਰਾ ਕਰਨ ਦੇ ਸਮਰੱਥ 19 ਲਾਈਨਾਂ ਨੂੰ ਚੁਣਿਆ ਗਿਆ। ਚੁਣੀਆਂ ਗਈਆਂ ਇਹਨਾਂ 19 ਲਾਈਨਾਂ ਵਿੱਚੋਂ, ਵਧੀਆ ਫ਼ਸਲੀ ਗੁਣਾਂ ਵਾਲੀਆਂ ਕੈਨਾਰੋਕਸਾ ਅਤੇ ਆਈ ਏ ਸੀ 47 ਲਾਈਨਾਂ ਨੂੰ ਕ੍ਰਮਵਾਰ ਪੀ ਆਰ 126 ਅਤੇ ਪੀ ਆਰ 116 ਨਾਲ ਕਰੋਸਿੰਗ ਲਈ ਡੋਨਰ ਲਾਈਨਾ ਵਜੋਂ ਵਰਤਿਆ ਗਿਆ। 78 SSR ਮਾਰਕਰਾਂ ਵਿੱਚੋਂ, RM16493, RM18457, RM25149 ਅਤੇ RM28404 ਨੇ ਕ੍ਰਮਵਾਰ ਗੁਣਸੂਰਤ 4, 5, 10 ਅਤੇ 12 ਉਪਰ, ਪੀ ਆਰ 116×IAC 47 ਵਿੱਚ ਬਹੁਰੂਪਕਤਾ ਦਿਖਾਈ। ਦੂਜੇ ਪਾਸੇ, ਪੀ ਆਰ 126 × ਕੈਨਾਰੋਕਸਾ ਦੇ ਲਿਹਾਜ਼ ਨਾਲ, RM104, RM289, RM7434, RM8243, RM264, RM25149, RM10 ਅਤੇ RM26643 ਮਾਰਕਰਾਂ ਨੇ ਕ੍ਰਮਵਾਰ ਗੁਣਸੂਤਰ 1, 5, 6, 8 ਅਤੇ 10 ਉਪਰ ਬਹੁਰੂਪਕਤਾ ਦਰਸਾਈ। ਮੈਪਿੰਗ ਜੰਨਸੰਖਿਆ ਵਿੱਚ ਅਨੁਵਾਂਸ਼ਿਕੀ ਵਿਭਿੰਨਤਾ ਦਾ ਪਤਾ ਲਗਾਉਣ ਲਈ ਇਹਨਾਂ ਬਹੁਰੂਪਕ ਮਾਰਕਾਂ ਦੀ ਵਰਤੋਂ ਕੀਤੀ ਗਈ। ਹਾਲਾਂਕਿ ਲੋਕਾਇ ਉਪਰ ਸਪਸ਼ਟ ਸੰਰਚਨਾ ਨਾ ਹੋਣ ਕਾਰਨ, ਇਸ ਅਧਿਐਨ ਦੌਰਾਨ ਪਹਿਚਾਣੇ ਗਏ ਬਹੁਰੂਪਕ ਮਾਰਕਰ, ਝੂਠੀ ਕਾਂਗਿਆਰੀ ਦਾ ਟਾਕਰਾ ਕਰਨ ਵਾਲੇ ਜੀਨ ਅਤੇ ਮਾਰਕਰ ਵਿੱਚ ਸਬੰਧ ਦਰਸਾਉਣ ਵਿੱਚ ਨਾਕਾਮ ਰਹੇ। ਅਗਲੀ ਪੀੜ੍ਹੀ ਵਿੱਚ ਮੁਲਾਂਕਣ ਕਰਨ ਨਾਲ ਝੂਠੀ ਕਾਂਗਿਆਰੀ ਦਾ ਟਾਕਰਾ ਕਰਨ ਵਾਲੇ ਜੀਨ ਅਤੇ ਮਾਰਕਰ ਵਿੱਚ ਸਬੰਧ ਸਥਾਪਤ ਕਰਨ ਵਿਚ ਮਦਦ ਮਿਲੇਗੀ।

ਮੁੱਖ ਸ਼ਬਦ: ਓਰਾਇਜ਼ਾ ਸਟੀਵਾ , ਝੂਠੀ ਕਾਂਗਿਆਰੀ, *Ustilaginoidea virens*, ਮੁੱਢਲੀ ਲਾਗ, ਪ੍ਰਤੀਰੋਧਕਤਾ, QTLs

ਮੁੱਖ ਸਲਾਹਕਾਰ ਦੇ ਹਸਤਾਖਰ **ਵਿੱਦਿਆਰਥੀ ਦੇ ਹਸਤਾਖਰ**

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CHAPTER I

INTRODUCTION

Rice (*Oryza sativa* L.) is a member of the family Gramineae (Poaceae). It is widely cultivated around the world and is important among the cereal crops. It is the staple food, especially in Asian countries and tropical Latin America. After China, India is the second largest producer of rice in the world with 177 million metric tonnes production contributing 26% in the world rice production (Anonymous 2020). It was cultivated on 162.06 million hectares of area with production 505 million metric tonnes around the world. In India, area under rice cultivation is about 44.5 million hectares accounting 40 per cent of food grains produced with annual production of 121.46 million tonnes (Anonymous 2020). The four states viz. West Bengal, Punjab, Tamil Nadu and Uttar Pradesh contribute chiefly to about half of the country's rice production. Besides wheat, this is also one of the important cereal crops in the north-western states of the country including Punjab.

Punjab is considered the "rice bowl of India" and is among the top ten rice growing states of India. In Punjab, rice is major *kharif* crop ranking second after wheat in terms of area, production and productivity. It occupies 3.1 million hectares with total production of 12.6 million tonnes of rice during the year 2019-20 (Anonymous 2020). The average yield of paddy was 59.61kg per hectare.

There are several diseases that affect the rice crop like sheath blight, blast, bacterial blight, false smut, stem rot, sheath rot which cause qualitative and quantitative losses. Among fungal diseases, false smut of rice caused by *Ustilaginoidea virens* (Cooke) Takahashi is an emerging disease. It was first reported from Tirunelveli district in Tamil Nadu (Cooke 1878). The teleomorph of the false smut fungus was *Claviceps oryzae sativae* (Hashioka 1971) but now, a new name *Villosiclava virens* has been proposed (Tanaka *et al* 2008). It is also known by other names as green smut or pseudo smut and is commonly called haldi disease, peeli ganth, peeli ulli and laxmi disease. It is becoming a serious disease and causing several losses in yield in major rice growing areas of the world.

The false smut disease was earlier believed to be a minor disease of rice (Ou 1985) but recently, the disease was found in epidemic form in various parts of Europe, Asia and America (Yaegashi *et al* 1989; Rush *et al* 2000; Tsuda *et al* 2006). The major cause of epidemics is widespread utilization of chemical fertilizers, high yielding varieties, high irrigation level along with favourable weather conditions (Wang *et al* 2004). The pathogen converts the kernel wholly or partially with mass of spores and gradually into smut balls. The sori of *U. virens* (Cooke) Takahashi, erupting through the lemma and palea, form a smut ball

of mycelia whose color changes gradually from yellowish green to dark green with maturity (Webster and Gunell 1992, Brooks *et al* 2009).

In India, the major rice growing states have suffered severe losses due to false smut ranging from 0.2 to 49 per cent in different regions and varies with different rice cultivars (Baruah *et al* 1992, Biswas 2001). About 10-20% incidence of false smut was recorded in Punjab on inbred rice varieties viz. PR116 and PAU 201. However, in southern states such as Tamil Nadu, disease incidence ranged from 5 to 85 per cent (Ladhalakshmi *et al* 2012). The yield losses due to this disease are also reported up to 44 per cent in Punjab (Pannu *et al* 2010).

The fungus, *U. virens* (cooke) Takahashi is also reported to cause false head smut in maize (*Zea mays* L.). This unusual disease has been observed in Gujarat state in 2013 (Gohel *et al* 2014). The pathogen is a member of *Ascomycete*. The fungus overwinters and survives by the means of chlamydospores and sclerotia that enable the pathogen to survive in the field for several months (Webster and Gunell 1992).

The smut balls produced by this pathogen on rice are covered by chlamydospres. These fresh thick-walled spores have high germination rate and cause new infection of the late maturing rice cultivars in the same year. The chlamydospres may contaminate the rice seeds or/and produces conidia which are air-borne and also spread by rain splash in the paddy fields where they overwinter when the conditions are unfavourable (Fan *et al* 2016). On the other hand, the fungus reproduces sexually by ascospores produced by sclerotia which serve as primary source of infection. During autumn, the low temperature favors the sclerotium formation from the smut balls. Sclerotia survive for 3-5 months in the paddy fields under low temperature and dry conditions while maintaining high germination rate up to 5 years (Yong *et al* 2018a). Under favourable conditions, (high humidity and moisture conditions) they germinate to produce stalked stromata full of perithecia at its apex. The perithecia produces large number of asci each containing eight ascospores. Later on, ascospores germinate to produce large number of conidia. Therefore, ascospores may serve as the primary inoculum as they are found primarily in the paddy fields for most of the rice growing period and may infect the rice panicles at boot stage causing primary infection of rice (Sun *et al* 2020, Yong *et al* 2018a). The fungus attacks weed species commonly occurring in the rice fields that may also serve as sources of inoculum (Atia 2004).

The symptoms produced by *U. virens* become visible only after flowering when the fungus attacks the ovaries of the rice kernels in its early development (Webster and Gunnell 1992). The symptoms appear in field during hard dough to mature stages of crop. The dynamics of the pathogen and the process of infection still remains unclear. The hypha

produced by the pathogen after germination enters rice spikeletes via a small gap between lemma and palea (Ashizawa *et al* 2012). Generally, only few or sometimes several grains of a panicle are affected. The fungus transforms individual kernels in the panicle or few spikelets in the panicle into a large, globose, velvety, yellowish spore mass (pseudomorphs). These balls are usually 2 to 5 cm in diameter and is larger than normal grains produced in the panicle. Initially, the spore mass or pseudomorph is light yellow and is covered by a thin orange membrane. Later on, the membrane bursts open and releases powdery mass of spores. The color of smut ball gradually changes to orange, yellowish green, green, olive, green and finally to greenish black. The pathogen produces ascospore as sexual spores and chlamydospores as asexual spores (Biswas 2001). The surface of the smut balls is covered by the powdery dark-green chlamydospores, conidia, mycelia and sometimes, even sclerotium (Ou 1972). The weed species that occur commonly in rice fields also harbors the fungus and may also serve as sources of inoculum (Atia 2004).

The incidence of false smut disease is favoured by high relative humidity (>90 %) (Bhagat and Prasad 1996, Yashoda *et al* 2000, Biswas 2001), moderate rainfall accompanied by cloudy days during flowering and temperatures between 25 and 30 °C (Chen *et al* 1994, Dodan and Singh 1996, Yashoda *et al* 2000). The studies on role of rainfall on development of false smut in rice yield ambiguous results. Both high and low rainfall at the heading stage of crop is known to increase the disease intensity (Sugha *et al* 1992, Dodan and Singh 1996, Cartwright *et al* 2002). High disease incidence (80-86%) was observed when artificial humidity was provided after inoculation of the rice plants with the false smut fungus (Pannu *et al* 2010). Late planting dates (Ahonsi *et al* 2000) and excessive application of nitrogenous fertilizers (Atia 2004) promotes the development of disease.

The disease cycle of this fungus-host interaction has not been completely characterized (Biswas 2001, Mew *et al* 2004, Fu *et al* 2012), but knowledge of the pathogen's life history and infection process is critical for efforts to control this disease (Tang *et al* 2013). Being a biotrophic pathogen, it hijacks the grain filling system of rice to meet its own nutritional requirements as well as imparting sterility to the surrounding spikelets. The pathogen is causing direct losses in grain yield of rice. The fungus also produces mycotoxins which are harmful to animal as well as human health, reducing the quality of grains unfit for consumption (Sun *et al* 2020).

The most economical, environmentally safe and efficient ways of crop protection is the utilization of resistant varieties in crop production. There are very limited sources of resistance to false smut and most of the cultivated varieties are susceptible to this emerging disease. Therefore, there is an increasing need to identify the potential donors from different germplasm lines of rice harboring immense genetic variability for resistance to false smut

(Hiremath *et al* 2021). In future, we can use these identified potential donors for transferring QTLs resistance to our cultivated and otherwise susceptible to false smut rice varieties.

Keeping in view the above-mentioned challenges, the present investigations were undertaken with the following objectives:

i. To understand the role of primary inoculum in false smut development

ii. To identify and validate QTLs governing resistance to false smut in rice

CHAPTER II

REVIEW OF LITERATURE

The literature pertaining to the present study "BIOLOGY OF *Ustilaginoidea virens* (Cooke.) Takahashi AND MAPPING QTL FOR RESISTANCE TO FALSE SMUT IN RICE (*Oryza sativa* L.) carried out at Punjab Agricultural University Ludhiana was reviewed under the following sub-headings:

2.1 Brief history, geographical distribution and economic importance

False smut is an emerging disease which is caused by *Ustilaginoidea virens* (Cooke) Takahashi. It was first reported from Tirunelveli district in Tamil Nadu (Cooke 1878). The teleomorph of the false smut fungus was *Claviceps oryzae sativae* (Hashioka 1971) but now, *Villosiclava virens* as a new name has been proposed (Tanaka *et al* 2008). It is also known by other names such as green smut or pseudo smut and is commonly called as haldi disease, peeli ganth, peeli ulli and laxmi disease. It has become a serious disease and causing several losses in yield in major rice growing areas of the world.

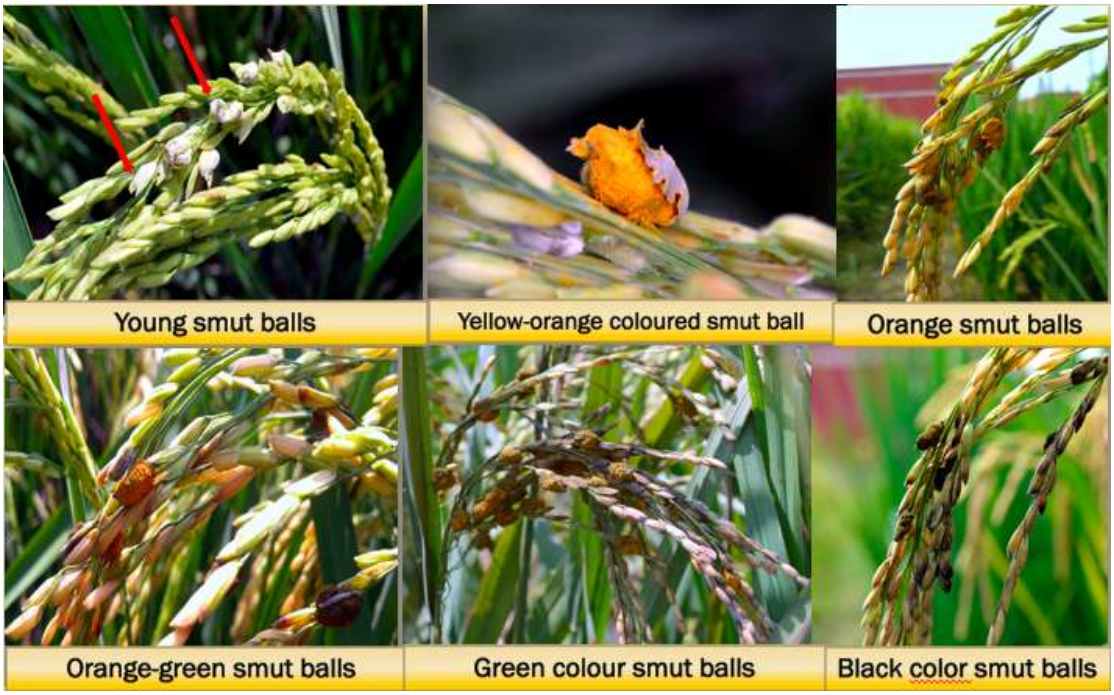
The false smut disease was earlier believed to be a minor disease of rice (Ou 1985) and it was regarded as a bumper/Lakshmi disease due to its high incidence under nutrient rich soil. The occurrence of false smut was also believed to be indicator of good crop year because of environmental conditions, particularly high humidity and rainfall, conducive for the disease development are also favourable for optimum rice production (Ou 1985). However, in recent years, the disease was found in epidemic form in various parts of Europe, Asia and America (Yaegashi *et al* 1989; Rush *et al* 2000; Tsuda *et al* 2006). The major cause of epidemics is widespread utilization of chemical fertilizers, high yielding varieties, high irrigation level along with climate change with increasing global temperatures (Wang *et al* 2004).

The incidence of false smut caused by *Ustilaginoidea virens* occurs in all the major rice growing countries of the world including, Peru, Australia, Bangladesh, Italy, Philippines, Myanmar, Fiji, Columbia, Japan, China, Thailand, USA, Brazil, Bolivia, Sri Lanka, Ghana, Indonesia, Ivory coast, Panama, Pakistan, Nigeria, Sudan, Tanzania, Trinidad, Vietnam, Venezuela, Zambia and in various American, Italian as well as South Asian rice-growing regions (Dodan and Singh 1996, Rush *et al* 2000). In India, the major rice growing states have suffered severe losses due to this disease since 2001. These different states include Haryana, Punjab, Uttar Pradesh, Uttaranchal, Tamil Nadu, Karnataka, Andhra Pradesh, Bihar, Jharkhand, Gujarat, Maharashtra, Jammu & Kashmir and Pondicherry (Dodan and Singh 1996, Mandhare *et al* 2008). The yield losses due to this pathogen ranges 0.2 to 49 per cent in different regions and varies with different rice cultivars (Biswas 2001). Since then the disease incidence occurs in moderate to severe form on commercial cultivars more or less every year. About 10-20% incidence of false smut was recorded in Punjab on inbred rice varieties viz. PR114, PR116 and PAU 201. However, in southern states such as Tamil Nadu, disease incidence ranged from 5 to 85 per cent (Ladhalakshmi *et al* 2012). The disease affects more than an area of 600 ha of rice in Raigarh district of Chhattisgarh in 2007 (Singh and Pophaly 2010). The losses due to this disease are also reported up to 44 per cent in Punjab (Pannu *et al* 2010).

The false smut causes both quantitative and qualitative yield losses in rice. The pathogen coverts the grains in rice spikelets into large, yellowish, velvety smut balls. The loss in yield of rice is attributed to the reduction in test weight, chaffiness in grains and sterility of the spikelets neighbouring the smut balls (Ladhalakshmi *et al* 2012). The chlamydospres produced by the pathogen consist of mycotoxins, ustiloxin and ustilaginoidin, which are harmful to animal as well as human health, reducing the quality of grains unfit for consumption (Koiso *et al* 1994, Zhou *et al* 2012, Sun *et al* 2020).

2.2 Symptoms of the false smut

It is also known by other names such as green smut or pseudo smut and is commonly called as haldi disease, peeli ganth, peeli ulli and laxmi disease. The characteristic symptoms produced by *U. virens* consists of transformation of grains in the spikelet into spore balls (Plate 1). Often, only few spikelets are affected in the inflorescence. The pathogen infects rice plant at booting stage asymptomatically and the visual symptoms appear in the form of smut balls only at during hard dough to mature stages of crop (Ashizawa 2012). The pathogen enters the rice spikelets through a small gap between lemma and palea where it develops asymptomatically. Later on, the fungus develops into massive mycelium and gradually attacks filaments of stamens and occasionally ovaries (Tang *et al* 2013, Hu *et al* 2014). Generally, only few or sometimes several grains of a panicle are affected. The fungus transforms



(a)

(b)

Plate 1: (a) The gradual symptoms of false smut caused by *Ustilaginoidea virens* (b) The incidence of false smut on susceptible check GSR123.

individual kernels in the panicle or few spikelets in the panicle into a large, globose, velvety, yellowish spore mass (pseudomorphs). These balls are usually 2 to 5 cm in diameter and is larger than normal grains produced in the panicle. Initially, the spore mass or pseudomorph is light yellow and is covered by a thin orange membrane. Later on, the membrane bursts open and releases powdery mass of spores. The color of smut ball gradually changes to orange, yellowish green, green, olive, green and finally to greenish black (Plate 1). The smut ball consists of three layers: outer yellowish green layer consists of mature chlamydopsores, middle orange layer consists of hypha and spores while the innermost whitish to light yellow layer consists of spores being formed (Ou 1985). The pathogen produces ascospore as sexual spores and chlamydospores as asexual spores (Biswas 2001). The surface of the smut balls is covered by the powdery dark-green chlamydospores, conidia, mycelia and sometimes, even sclerotium (Ou 1972).

2.3 Etiology

Kingdom : Fungi

Division : Ascomycota

Class : Sordariomycetes

Order : Hypocreales

Family : Clavicipitaceae

Genus : *Villosiclava*

Species : *virens*

The false smut of rice is caused by an ascomycete *Ustalginoidea virens* and not basidiomycete (true smut). The teleomorph of fungus had been named *Claviceps virens* (Sakurai ex Nakata) and *Claviceps oryaze sativae* (Hashioka) as its teleomorphic characteristics resembles to *Claviceps* (Hashioka 1971). The members of Ustilaginoideae were found to be closely related but distinct from those of teleomorphs of Clavicipitaceae on the basis of molecular phylogenetic analysis using sequences of the large subunit of the ribosomal RNA gene. Therefore, it was suggested that members of Ustilaginoideae should be regarded as a monophyletic group within the order Hypocreales based on ALDH-1 gene. The members of Claviceps encode ALDH-1 gene, a member of the aldehyde dehydrogenase family (Bischoff *et al* 2004, Tanaka and Tanaka 2008). As a result, *Villosiclava virens* was suggested as the new name for the teleomorph of *U. virens* (Tanaka et al 2008).

The anamorph and teleomorph stages of pathogen consists of *Ustilaginoidea virens* and *Villosciclava virens* respectively. The fungus produces three types of spores chlamydospores, ascospores as well as conidia. It is heterothallic and mating compatibility is

identified on the basis of MAT-1 and MAT-2 of mating type locus-1. The fungus is identified by the chlamydospores which are assembled on the spore balls. Spore balls consists of radial, branched and compacted hyphae consisting of spherical to elliptical (3-5 × 4-6 μm), warty chlamydospores at terminus (Ou, 1985). The chlamydospores are round to elliptical, smooth when immature but gradually, becomes warty, spiny and yellow to orange pigmented following maturity (Kim and Park 2007). In culture, the chlamydospores germinate by fine

germ tubes giving rise to 1- 3 small ovoid secondary conidia (Hashioka *et al* 1951). The fungus is slow growing and can be cultured on potato dextrose agar (PDA) medium and produces two types of colonies. It produces white fluffy mass of mycelium consisting of conidia after 7-10 days of isolation from the smut balls collected from the previous year. The other colony appears later after 15 days of isolation consisting of chlamydospores which are green and hard. The chlamydospores, produced on the surface of smut balls of rice, germinates and produce conidia to cause secondary infection of rice. The chlamydospores serve as the important source of inoculum between the seasons of rice (Zhang *et al* 2003).

The sexual stage consists of overwintered sclerotia which are produced under low temperature later in autumn on the surfaces of rice false smut balls. Under favourable conditions of moisture, light and temperature, sclerotia in the field germinates in summer to produce stalked stromata (ascomata) with perithecia at its apex. The perithecia consists of numerous arranged asci bearing ascospores (Ou 1985, Lee and Gunnell 1992, Tanaka *et al* 2008, Yu *et al* 2015). The ascospores produce secondary conidia to cause primary infections of rice at booting stage (Yong *et al* 2018a).

2.4 Disease cycle of false smut: confusion and uncertainty

The disease cycle of *U. virens* is poorly understood but knowledge of the history, life cycle and infection process is crucial for development of disease management strategies against false smut of rice.

U. virens is non-obligate biotroph. It can be cultured under laboratory conditions and reproduce sexually and asexually only on a living host. The symptoms produced by *U. virens* become visible only after flowering when the fungus attacks the ovaries of the rice kernels in its early development (Webster and Gunnell 1992). The pathogen produces ascospores as sexual spores and chlamydospores as asexual spores (Biswas 2001). The surface of the smut balls is covered by the powdery dark-green chlamydospores, conidia, mycelia and sometimes, even sclerotia (Ou 1972). The pathogen infects rice flowers and produce large number of chlamydospores. These fresh thick-walled spores have high germination rate and cause new infection of the late maturing rice cultivars in the same year. The chlamydospores may contaminate the rice seeds or/and produces conidia which are air-borne and also spread by

rain splash in the paddy fields where they overwinter when the conditions are unfavourable (Fan *et al* 2010, Fan *et al* 2016). The chlamydospores germinate under high moisture conditions giving rise to conidia which infects rice at the booting stage.

The sexual stage consists of overwintered sclerotia which are produced under low temperature later in autumn on the surfaces of rice false smut balls (Qiu *et al* 2019). Under favourable conditions of moisture, light and temperature, sclerotia in the field germinates in summer and produce ascospores (Tanaka *et al* 2008, Yu *et al* 2015). The teleomorphic stage of the fungus is rarely reported under Indian conditions due to absence of its mating types *viz.* MAT1 and MAT2 as the fungus is heterothallic (Qiu *et al* 2019).

The disease cycle of the pathogen and the source of primary inoculum still remain ambiguous. It has been hypothesized that sclerotia act as the primary source of inoculum of this disease rather than chlamydospores. The ascospores are usually found before and after rice planting in the paddy fields suggesting that they are being produced regularly by the overwintered sclerotia from May to September, the critical period for infection by the pathogen. On the contrary, chlamydospores are only found in the form powdery mass on the smut balls on rice (Yong *et al* 2018a).

There are two school of thoughts behind the disease cycle of this pathogen. First, the primary source of infection may be caused by the conidia produced by the chlamydospores or/ and ascospores produced by the overwintered sclerotia during favourable warm temperatures. They both germinate and land on the inflorescence of rice during the boot stage of rice. Secondly, the fungus overwinters by means of sclerotia in soil or debris and/ or as spore balls in seed and/or soil. The spore ball germinates and give rise to chlamydospores. The hyphae from the chlamydospores colonize seedlings coleoptile and gradually, the apical meristem of the rice tissues. Later on, the fungus colonizes rice flowers prior to emergence of the panicle (Ou 1985, Sreeramulu and Vittal 1996, Biswas 2001, Zhou *et al* 2003, Ashizawa and Kataoka 2005, Schroud and TeBeest 2005, Zhou *et al* 2008, Qiu *et al* 2019). Ditmore and TeBeest (2006) and TeBeest *et al* (2010) reported that increasing the *U. virens* inoculum levels in the soil increases the infection levels of rice.

TeBeest *et al* (2010) also found that the fungus is soil-borne in the form of spore balls found in the field heavily infested with false smut. The healthy rice seeds planted in a field heavily infested with false smut and found that 75% rice seedlings were infected by the pathogen within three weeks after emergence from the soil as tested by PCR analysis. The study was further corroborated by the evidence that the rice panicles were neither inoculated at flowering with spore suspension of *U. virens* nor the field under the test was located near the rice fields. Therefore, eliminating the other sources of infection of rice plant by the

pathogen. These findings are in accordance with the above results of the current research that the fungus is soil-borne in nature and later on it invades the seedlings of rice.

Ashizawa *et al* (2010) detected chlamydospores in the soil by quantitative measurement with the help of real-time PCR approach with species specific primers. The study provides an efficient evidence about the role of soil-borne chlamydospores in causing primary infection of the rice plants. TeBeest *et al* (2010) reported that increasing the *U. virens* inoculum levels in the soil increases the infection levels of rice.

Tanaka *et al* (2017) also found that the fungus is found in the soil in the form of thick-walled conidia and nested PCR species-specific primers reveals the presence of *U. virens* in rice roots and apices at vegetative stage of plant.

Besides rice, the alternative hosts of *U. virens* includes several weed species *viz. Digitaria marginata*, *Panicum trypheron*, *Echinochola crusgalli* and *Imperata cylindria* suggesting the role of weeds in the growth of pathogen and disease cycle (Atia 2004).

2.5 Infection process of *U. virens*

The infection process of pathogen was elucidated with the green florescent protein labelled strain (Ashizawa *et al* 2012, Tang *et al* 2013, Hu *et al* 2014). The *V. virens* infects rice flowers and spores germinate on the developing spikelet. The hyphae without penetrating the spikelet and spread over the inner surface of the spikelet via a small gap between lemma and palea without infecting it. After entering, the pathogen preferentially infects filaments of stamen between the lodicules and ovaries without formation of haustorium or appresorium (Tang *et al* 2013, Fan *et al* 2020). Gradually, the hyphae grow profusely to form massive mycelium and encloses the anthers, stigma, ovaries and lodicules at 10 days post inoculation. The mycelium then protrudes out of the spikelets in the form of smut balls consisting of chlamydospores on the surface of the balls at 15 days post inoculation. The pathogen inhibits the pollination and mimics the fertilization process of ovaries in order to obtain continuous supply of nutrients without killing them. This is characteristic feature of the biotrophic pathogen (Tang *et al* 2013, Fan *et al* 2015, Song *et al* 2016, Qiu *et al* 2019).

Apart from spikelets of rice, *U. virens* also infects other rice organs without producing any disease symptoms. The pathogen propagules can germinate on the surface of rice coleoptile after germination of the rice seeds as well as on the surface of the roots at the seedling stage of rice (Raychaudhari 1946, Ikegami 1963, Schroud and TeBeest 2005, Prakobsub and Ashizawa 2017, Yong *et al* 2018b, Qiu *et al* 2019). However, hyphae fail to penetrate the sclerenchyma layer and reach the endodermis as well as vascular tissue in order to cause systemic infection of rice. Moreover, *U. virens* has been found absent in vascular tissue of infected plant (Tang *et al* 2013, Yong *et al* 2018b). In

addition, no appresorium or haustorium was detected in infected roots or coleoptiles (Yong *et al* 2018b).

Zhou *et al* (2006) and Tanaka *et al* (2017) reported that chlamydospores can travel all along from the infected root or coleoptile and gradually cause flower infection at the heading stage of rice. The recent PCR/ colorimetric *in situ* hybridization approaches can be used to detect the presence of pathogen from vegetative to heading stage of rice.

2.6 Isolation, culture and identification of *U. virens*

The isolation of *U. virens* is very tedious as this is very slow growing pathogen and require specific substrate for its viable growth and sporulation. The pure culture of the pathogen is usually contaminated by various fast growing saprophytes during incubation. The isolation of the pathogen in its pure form is crucial to study various aspects of the disease *viz.* morphological and cultural variability, disease cycle, standardized inoculation technique, screening varieties for resistance etc. It takes about 20-25 days for obtaining axenic culture of the pathogen.

Brefeld (1895) reported that the pathogen can be cultured artificially from conidia or sclerotia in the form of white velvety mycelium with numerous spores which later on turns dark green. Sharma and Joshi (1975) used sclerotia for producing conidia of *U. virens* on yeast peptone potato dextrose agar (YPPDA) medium. The same medium was used with 1ml of kinetin solution to produce artificial chlamydospores on discs of sterilized butter paper. Moreover, smut balls were also produced artificially on sterilized detached rice florets which were smaller than that produced under natural conditions (Singh and Gangopadhyay 1981).

The maximum growth of the fungus was supported by maltose and asparagine (Singh and Verma 1981). The chlamydospores germinate on sterilized water and water agar at 25°C and pH 5-8 (Tsai *et al* 1990) whereas conidia germinate best on potato decoction or sugar solution (Hashioka *et al* 1951). The production of conidia in axenic culture depends upon various factors such as temperature, substrate as well as the incubation time the isolate is in culture Wang (1988) used potato sucrose broth (PSB) medium and potato dextrose broth (PDB) medium for production of up to 10^8 conidia/ml of broth in just 6 days. However, PDB supplemented with 2% sucrose along with barley seeds produced more conidia in less time than other media (Ashizawa *et al* 2011). The conidia are produced in large amounts after shaking at 140-200 rpm at 25-27°C for 7 days (Shi *et al* 2017). The production of conidia on media largely depends upon the number of transfers as well as storage time for which the culture remains in laboratory without sub-culturing. The conidia capacity and viability of conidia reduces with repeated and delayed sub-culturing. Wang *et al* (2019) used rice leaves and panicle filtrate medium to induce conidiation of *U. virens* with leaf media inducing more

conidiation. Although, it produced smaller conidia but could infect rice after culturing them on potato sucrose broth (PSB) for 8-12 h with shaking.

The culture of *U. virens* was also obtained on XBZ medium and transferred into PSB at 26°C for inoculation of rice by boot injection method (Zhou and Zhang 1999). Haiyong (2012) found that PSB medium produce higher concentration of conidia 7.25×10^{7} conidia ml^{-1} out of seven media used in the study. The study also standardized single spore isolation technique and yellow colour smut balls were considered more reliable for isolation of the pathogen. Fu *et al* (2013) also found PSA medium as the best for mycelial growth and sporulation. Among the nutrients, the study found sucrose and starch as the best carbon sources while ammonium chloride, ammonium nitrate and ammonium sulphate as best nitrogen sources for mycelial growth of fungus. The mycelial growth was found maximum under dark condition as fluorescent light was inhibitory to its growth.

Lu *et al* (2009) used wakimoto toceshi (XBZ) medium (Zhou and Zhang 1999) and potato sugar (PS) liquid medium for isolation of *U. virens* and conidial production respectively in an experiment testing pathogenicity of isolates. The study used 75% ethanol for 2-3 minutes followed by 0.1% mercuric chloride for 1-3 minutes for surface sterilization of the smut balls.

The germination of the chlamydospores in medium after isolation was best from the fresh samples and germination percentage of the spore decreases gradually with time of storage (Huang *et al* 2010, Baite and Sharma 2015).

Baite and Sharma (2015) standardized the isolation technique for *U. virens.* Out of three media used in study *viz.* potato dextrose agar, rice yeast extract dextrose agar (RYDA) and potato sucrose broth (PSA), the best growth of the fungus was found on PSA medium for most of the isolates tested. The optimum growth of the fungus was found after incubation under dark at 25°C and pH-6. The study also found that the surface sterilization of the smut balls with 1% sodium hypochlorite solution for 1 minute followed by 70% ethanol for 1 minute gave best results as compared to earlier described sterilization techniques.

U. virens on PSA medium produce creamy white, compact and fluffy colony after 6-7 days of isolation. Gradually, the colony changes to yellow colour producing chlamydospores in the centre then appear dark to olive green as the culture matures after 15 days of isolation (Baite and Sharma 2015). The characteristic transformation of the colony colour from white to olive green on the medium resembles that of the smut balls on the rice panicle under field conditions. (Ladhalakshmi *et al* 2012).

The fungus produces yellowish brown spherical to elliptical, double-walled chlamydospores borne laterally on streigmata. These thick-walled spores germinate by short

germ tubes and give rise to conidiophores. The conidia are minute, hyaline, ovoid to elliptical borne at the tapering apex of conidiophores (Ladhalakshmi *et al* 2012).

2.7 Factors related to pathogenicity

The occurrence of false smut of rice is related with high humidity (>90%) and rainfall during flowering under highly fertile soil conditions (Ahonshi *et al* 2000). Low temperature exposure after inoculation has a strong stimulatory effect on disease development (Fujita *et al* 1989, Ladhalaxmi *et al* 2012).

The germination of chlamydospores occur at 28°C under high humidity and moisture conditions (Lu *et al* 1996). The sclerotia germinates only after entering the period of winter dormancy for 6-7 months below 20°C. After a period of dormancy, it germinates at 26-28°C and under high moisture conditions (Dong and Fu 1989, Huang *et al* 2019). The optimum temperature for the germination of conidia was found between 28-30°C with minimum wetness period of 2 hours (Fu *et al* 2013).

In a two-year survey conducted by Atia (2004) on yield losses of rice, it was found that early maturing rice varieties showed less disease incidence of false smut as compared to late maturing rice varieties. Moreover, the disease incidence was more in case of soils rich in nitrogen fertilization, sandy or loose soils and late transplanting of rice. The early maturing rice genotypes escape disease because relative humidity and temperature are higher later in the season which is highly conducive for the development of the pathogen (Nessa *et al* 2015).

In an epidemiological study of false smut in Bihar State, maximum disease incidence and severity were recorded at temperature 24-32°C, relative humidity 74-88%, rainfall 6.66mm and 6.2-6.29 hrs sunshine (Bhargava *et al* 2018). The severity of disease was high in Rice-crayfish co-culture due to high relative humidity and moderate temperature conditions in above system than in rice monoculture (Jiehui *et al* 2021). Rainy days during the heading stage of rice increases the incidence of disease (Wu *et al* 2000). Moreover, high relative humidity and wetness period during heading of rice is also very critical for pathogen infection (Fan *et al* 2014, Jia *et al* 2015). The temperature and humidity are more important for the disease development than concentration of the conidia. After artificial inoculation of conidia by boot injection, the inoculated plants should be placed at 16°C for 2 days followed by 26°C with 100% humidity for 5 days for the best development of the false smut balls (Ashizawa *et al* 2011). The environmental factors that rice plant encounter during its most susceptible stage (late booting and beginning of heading to flowering) decide the fate of disease development and severity. If this stage encounters heavy rainfall with more rainy days, high relative humidity (>85%), short sunshine hours, suitable temperature (22-28°C) with smaller

difference between day and night temperatures then the incidence of false smut will be more severe (Fei *et al* 2010, Kumar *et al* 2020).

The late planting of late maturing varieties as well as early planting of early maturing varieties should be avoided as they are more vulnerable to the infection by the pathogen. Lore *et al* (2021) evaluated three planting dates for the incidence of false smut disease under different planting dates. The disease incidence was found to be higher in early planting dates (June 25) than at second (July 5) and late planting date (July 15). This was because the weather conditions such as temperature (25.2-31.7°C), rainy days (7 days) and relative humidity (73-92.5%) were highly favourable for the disease development. The study also found the cultivar HKR 47, pure-line cultivar, showed lowest disease incidence at all planting dates. The early transplanted rice (June 25) shows higher incidence and severity of false smut than in late transplanted rice (July 25) under Indian conditions (Dodan and Singh 1996, Bhargava *et al* 2018).

On the other hand, early planting lowers the disease incidence as compared with late planting in Nigeria, United States, Egypt, Eastern India, and Bangladesh (Ahonsi *et al* 2000, Cartwright *et al* 2002, Atia 2004, Bag *et al* 2016, Sarker *et al* 2016). The coincidence of favourable weather factors later in the season are highly conducive for the pathogen to germinate and colonize the rice plants. Meanwhile, the crop is at booting stage which is the most susceptible stage of rice which renders the plant susceptible to attack by the pathogen. Lore *et al* (2021) also found that weather factors such as rainfall, relative humidity and rainy days during the flowering of rice plays an important role in the disease development. The interaction of host-plant as well as environmental factors play a key role in development of disease. Therefore, the disease can be avoided by the manipulation of the planting date of rice cultivars.

2.8 Nested PCR approach for asymptomatic detection of *U. virens* in rice tissues

Molecular biology tools such as polymerase chain reaction have made it possible to detect and quantify micro-organisms in the susceptible plants asymptomatically. In case of false smut of rice, symptoms appear only after flowering and management of this disease on the basis of its diagnosis is too late to avoid economic yield loss. The detection methods of the disease based on the morphological examination of the culture after isolation of the pathogen in its pure form is very tedious and non-specific. Therefore, there is need of specific and sensitive detection assays for the asymptomatic identification of *U. virens* in rice tissues. This knowledge will open door for our better understanding on ambiguous life cycle of the pathogen and epidemiology of the disease.

Molecular biology tools such as polymerase chain reaction (PCR) have been used effectively to detect *U. virens* spores in asymptomatic rice plants as well as grains (Chen et al

2014, Zhou et al 2003). Moreover, correlation between the amount of *U. virens* in the soil and the prevalence of rice false smut disease in rice plants has been ascertained using real-time PCR (qPCR) (Ashizawa et al 2010). Chen *et al* 2014 detected specific primers for detection of *U. virens* in rice seeds.

To date, three PCR-based protocols have been published for the detection of *U. virens* (Zhou *et al* 2003, Zhou *et al* 2008, Ashizawa *et al* 2012), all of which target the rDNA sequences of the ITS region in the pathogen's genome. These sequences were used because of their high copy number and heterogeneity of their non-coding ITS region (White *et al* 1990). Several studies have relied on the nested PCR approach (Zhou *et al* 2003, Tang and Zheng 2017) to analyze the possibility of systemic infections (Ditmore and TeBeest 2005, TeBeest *et al* 2010).

Zhou *et al* (2003) compared microscopy and PCR based method for asymptomatic detection of infection by *U. virens* and *Ephelis japonica* in rice tissues. After artificial inoculation by boot injection method and incubation of rice plants (15°C for 2 days and subsequently at 26°C for 5 days), the asymptomatic rice tillers were sampled prior to booting stage for microscopic examination and PCR detection. They used US1-5, US2-5, US3-3 and US4-3 primers for the study. They used the primer pair US1-5/ US3-3 for simple PCR and then used its PCR product for nested PCR with the primer pair US2-5/ US4-3. The primer pair US1-5/ US3-3 and US2-5/ US4-3 gave 380bp and 232bp PCR product, respectively. They used the primer pair US1-5/ US3-3 for simple PCR and then used its PCR product for nested PCR with the primer pair US2-5/ US4-3. They found that the nested PCR has 1000 times more sensitivity than simple PCR. After histological examination of inoculated rice plants under compound microscope, they observed conidia and hyphae of *U. virens* on inner sheath at injection sites as well as on spikelet after 7 days of inoculation. Furthermore, nested PCR produced bands and detect the fungus from otherwise non-obvious fungal infection under microscopy. Therefore, PCR protocol can detect even latent infection by *U. virens* and *E. japonica* in the rice tissues.

Li *et al* (2014) has found real-time PCR assay more sensitive and reproducible than conventional nested-PCR assay in detection and quantification of *U. virens*. Tang and Zheng (2017) detected six sets of nested PCR primers which have high sensitivity and specifity against target unique genes of *U. virens* which could be used as primers for species specific detection of *U. virens* in symptomless rice plants. The nested PCR primer was designed from whole genome sequencing rather than ITS spacer region (Ashizawa *et al* 2012, Chen *et al* 2014, Li *et al* 2014).

Tang and Zheng (2017) used primer sequences mined from whole genome sequences and using comparative genomics for selection of target genes. Out of 96 unique genes of *U.*

virens identified through genome comparisons, they chose randomly 20 candidate *U. virens*-specific genes for nested PCR detection. To access the specificity of the above selected primers, they tested 97 *U. virens* strains, 16 other fungal pathogens, two bacterial pathogens and two rice species. Out of 20, 19 nested PCR primer set was able to produce amplicon with genomic DNA of 97 *U. virens* strains resulting in 200-800bp amplicon while no products were obtained from 16 other fungal pathogens, two bacterial pathogens and two rice species. Among 19 sets of nested PCR primers giving results, 6 sets were found to be highly sensitive and specific for the target regions G544, G858, G924, G1384, G1581 and G5140 which can be used as species specific primers for *U. virens* detection.

2.9 Artificial inoculation of *U. virens* in rice

The evaluation of resistance against false smut in rice is very important for identification of potential resistance sources. The evaluation of rice accessions naturally under field conditions depends upon various environmental conditions for the development of this disease (Kurauchi *et al* 2006). Thus, we have limited resistant sources available against this disease (Raji *et al* 2016, Hiremath *et al* 2021). A standardized artificial inoculation technique would help in large scale screening of rice genotypes and impart us knowledge about resistance of rice to false smut, disease cycle and infection process of the pathogen.

The disease is produced artificially by spray or boot injection of conidia, chlamydospores or even ascopsores at the boot stage of rice. The spores are believed to germinate and land on the inflorescence of rice during the boot stage of rice implying that the boot stage or flowering stage is the most susceptible stage for infection by this pathogen (Ou, 1985, Biswas 2001, Ashizawa and Kataoka 2005, Schroud and TeBeest, 2005, Zhou *et al* 2003, Zhou *et al* 2008). The infection of pathogen might also be occurring at the flowering stage of rice (Butler 1918).

The disease was developed artificially in Japan by boot injection method. The researchers injected spore suspension into the leaf sheath at booting stage of rice (Yoshino and Yamamoto 1952). Ikegami (1960) used chlamydospore suspension for inoculation of rice by same method.

In a study by Kulkarni and Moniz (1975), the ovaries of rice were artificially infected with spore suspensions of chlamydospores with the help of camel-hair brush, successfully producing about 80% infection of rice panicles. However, seed inoculation by smearing seeds with chlamydospores does not produce any infection. The results of this study imply that the infection is either air-borne and/or soil borne but rarely seed borne. The chlamydospores from the soil gave rise to conidia which are dispersed by wind and/ or rain splashes. The same study was conducted by Singh and Gangopadhyay (1981), used sterilized camel-hair brush for inoculation of chlamydospores on rice flowers. Singh and Dubey1984 was also able to

produce false smut disease successfully by spraying spore suspension of fungus in the morning hours when the rice florets were open. He also used several other methods in his experiment which includes dusting of conidia on open florets, infecting fertilized ovaries by conidial suspensions with the help of camel-hair brush, spraying spore suspension in the morning, injection of spores into the leaf sheath. Li *et al* (1986) conducted an experiment to uncover the susceptible stage of infection by *U. virens* by inoculating at the pathogen at various stages of rice plant. They found that the infection occurred at late booting stage of rice. Dhindsa (1988) used some new methods of inoculation in addition to those used by Singh and Dubey1984. He inoculated the rice panicle by dipping them in spore suspension of the fungus, spraying of suspensions prepared from mycelial bits as well as modified Gogo method. Although, he could not get success in producing disease through artificial inoculation.

The most widely accepted and effective method of inoculation was developed by Fujita *et al* (1989). They used conidial pure culture of *U. virens* on potato sucrose agar for preparing suspension of conidia. The conidial suspension (1×10^6 conidia/ml) of 2ml was used for injection into leaf sheath of rice at booting stage. The inoculated plants were placed at 15°C for 2 days and afterwards, at 26°C for 5 days in moist chamber to maintain relative humidity. Subsequently, the plants were incubated at 25-35°C in a greenhouse until the appearance of symptoms on panicles. The study found that the low temperature (15°C) and high humidity (100%) are essential for infection by the pathogen while high temperature is required for the development of balls on the panicle. This study was the first ever experiment for the artificial inoculation of conidia and creation of epiphytotic conditions for the successful development of false smut.

Liao and Wang (1994) developed method for artificial screening of rice genotypes against false smut of rice. The study used two methods of inoculating *U. virens*, one by spraying conidial suspension at 7-15 days before rice ear emergence stage and secondly, dusting of chlamydospores in the soil around the plants.

Wang *et al* (1996) found conidial injection method of inoculation into the leaf sheath was more producible as compared to spray inoculation as the disease incidence was more in former than latter. The study also concluded that the boot stage of rice was most susceptible stage of infection by *U. virens* and potato extract in the inoculum increases the disease severity.

Hedge *et al* (2000) concluded in an artificial inoculation experiment that the false smut of rice may be air-borne and rarely seed as well as soil borne disease. The spraying of *U. virens* chlamydospore suspension at flowering stage of rice showed maximum disease development (20%). However, no disease symptoms were produced when the seeds from

infected panicle from previous season were used for sowing. Similarly, disease incidence was absent when seeds and roots of the rice seedling were dipped in chlamydospore suspension were used for planting. The same results were produced by the similar study conducted by Yashoda and Anahosur (2000).

Zhang *et al* 2003 reported 100% infection of the rice panicles by boot injection method. They used mixture of hyphae and conidia from pure culture of *U. virens* for injection into the leaf sheath of rice at boot stage.

The standardization of technique for artificial creation of false under field conditions was done by Pannu *et al* (2010). They used PR116 plants which was susceptible variety to false smut in Punjab. The yellow spores from the freshly collected smut balls of diseased plants were used in the form of spore suspension for spray inoculation of PR116 at boot stage. The plants were sprayed with water in the field using perfo-spray system at hourly intervals during the daytime for 15 days for maintaining necessary relative humidity for the disease development. The study concluded that artificial humidity was necessary to be maintained for at least 15 days after inoculation as evident from the results. The disease incidence was more pronounced in plants which were given high humidity treatment as compared to untreated plants.

A method used by Ashizawa *et al* (2011) for evaluating false smut resistance in 18 varieties/ lines includes cultivation of the main culm of the rice plant and standardization of conidial spore suspension for inoculation by boot injection. The main culm of the rice plant was allowed to grow under greenhouse conditions by removal of the tillers of the plant periodically. They developed a faster method for producing quantity of conidia which includes incubation of either four barley seeds for 4 days or eight barley seeds for 3 days inoculated with *U. virens* in potato dextrose broth containing 2% sucrose. The conidial suspension of 2 ml was inoculated at the boot stage of the crop at different concentration of 5, 25, 50, 75 × 10^5 conidia/ml. The inoculated plants were incubated at 16°C for two days and then transferred to a moist chamber maintained at 26°C and 100% relative humidity for five days (Guo *et al* 2012). Subsequently, the plants were incubated in greenhouse at 25/20°C (day/night) until the development of ball like symptoms of false smut. The conidia injected with concentration of 5×10^5 conidia/ml results in higher disease incidence than other concentrations while conidial concentration of 7.5×10^5 conidia/ml causes sterility of panicles and fails to head.

Haiyong (2012) used spore suspension of 2008-33-1 *U. virens* isolate for evaluation of two inoculation methods of false smut in rice variety Gangxing 707. They carried inoculation at three different growth stages of rice with conidial injection and the other with conidial spray. They found that crop inoculated at boot stage produced highest false smut incidence (50.43%) with conidial injection than spraying suspension of conidia (34.75%). The

above results implied that for higher disease incidence, the suspension of conidia should be injected into the leaf sheath of rice at the late booting stage of crop (Haiyong *et al* 2015).

A successful method of artificial inoculation was developed by Ladhalakshmi *et al* (2012) for testing pathogenicity on paddy variety TN-1 under greenhouse conditions. They made slight modifications in method as used by Fujita *et al* (1989) by injecting rice plants with 2ml of conidial suspension (2.5×10^5 conidia/ml) of *U. virens* at boot stage. The inoculated plants were kept in humidity chamber maintained at 95% relative humidity and thereafter at 27°C under greenhouse. The plants injected with sterile water were served as a control. The false smut symptoms in the form of smut balls on rice panicles were observed 15 days after the inoculation of plants.

Tang *et al* (2013) filtered conidia from hyphae by centrifugation. The fresh potato sucrose medium was used to re-suspend the filtered conidia at a density of 2.5×10^5 conidia/ml. The above inoculum was used for inoculating plants which were maintained in a growth chamber illuminated with white fluorescent tubes with 85-95 per cent relative humidity at 20°C for two days. This resulted in increased infection of the plants.

Hu *et al* (2014) inoculated rice panicle at the booting stage under greenhouse to study the relationship between the artificial inoculation and disease incidence. They used different volume (0.2, 0.5, 1 and 2ml) of a mixture of hyphae and conidial suspension (2.5×10^5 conidia/ml) which were inoculated at apex, mid-point and base of rice panicles. They found that increase in volume of inoculum causes more severe infections and the optimum inoculum was 1-2ml inoculated at the mid-point of the panicle.

Hiremath *et al* (2021) screened 125 rice accessions from global rice diversity panel for identification of potential donors for false smut resistance. They screened the accessions under natural hotspot for three consecutive years as well as under artificial inoculation conditions in the fourth year using spray inoculation technique. The single spore was used for obtaining pure culture of *U. virens* and spore suspensions was prepared in sucrose broth. The germplasm lines were sprayed at booting stage of plant with spore suspension containing concentration of 7.5×10^5 conidia/ml. The plants were incubated at 90 per cent relative humidity with maximum and minimum temperature of 30±2°C and 24±2°C respectively. Out of 125 germplasm lines, 25 lines were highly resistant in all the four years under natural as well as artificial conditions whereas 31 lines were moderately resistant to false smut.

2.10 Screening for resistance to false smut of rice

The reports on rice resistance to false smut is very limited (Guo *et al* 2012). The major drawbacks in screening of rice diverse sources against this disease were due to its minor importance in the past decades, lack of standardized inoculation technique and

difficulty in obtaining axenic culture of the pathogen for artificial inoculation. The screening of rice germplasm lines against this disease is done through artificial inoculation by boot injection method. This method is not only tedious and needs technical expertise but requires certain controlled conditions for the disease development. The screening of rice accessions naturally depends upon various environmental conditions for the incidence of this disease under field conditions. Thus, we have limited resistant sources available against this disease (Hiremath *et al* 2021, Raji *et al* 2016). However, the natural hotspots of disease allow us to screen our large breeding population along with artificial inoculation of selected lines would help us to identify resistant sources of rice against the false smut. A wide variation in the resistance of rice genotypes against the false smut of rice has been reported (Anand *et al* 1985, Singh *et al* 1987, Singh and Kang 1987, Singh and Khan 1989 and Sugha *et al* 1992, Kurauchi *et al* 2006, Lore *et al* 2013).

In a screening experiment, 91 rice lines were found to be resistant while 4 lines were susceptible to false smut disease (Ashrafuzzaman 1974). In an experiment on disease resistance of rice to false smut by Ahonsi *et al* (2000), wide variation in the rice resistance to false smut was reported. IRAT 170 reported high level of resistance to false smut and ITA 316 and Ex- China were moderately resistant while Agbede, ITA 150, ITA 315, and ITA 335 were found to be highly susceptible to false smut. Biswas (2001) evaluated the resistance level of 41 rice hybrids to false smut as well as kernel smut of rice under field conditions. Among 41, eight rice hybrids were found completely free from the false smut disease.

In a study by Hedge (1998), screening of 58 rice lines resulted in selecting 15 genotypes free from infection while remaining showed 2-20% infection by the pathogen. These 15 lines includes IR 60, IR 66, IR 30864, Kavya, IESHI, IESH 2, IESH 3, PNR 162, R 51-91-7, IR 62, IR 64, K-44-1, Bulbuni, Jasmine 85 and Manila. In a screening experiment of rice varieties against false smut under rainfed and upland conditions, among seven genotypes only two were found to be resistant. These highly resistant varieties include IRAT 170 and Ex-China.

Dodan and Singh (1996) evaluated various scented and non-scented rice varieties for identification of resistance against false smut. Among 143 rice genotypes, only 20 of them exhibited false smut resistance. Similarly, another experiment screened five commercial rice varieties during 2000 and 2001 under field conditions in Egypt. These varieties include Giza 178, Sakha 102, Sakha 101, Giza 171 and Riho. Among these varieties, Giza 171 was found to be highly susceptible to false smut as this is long duration variety. On the other hand, the short duration varieties (Sakha 101, Sakha 102) showed lower disease incidence. This concludes that the short duration rice varieties escape the disease by escaping the conducive environmental conditions at the susceptible stage of rice to false smut (Atia 2004). Singh and

Singh (2005) found 27 rice genotypes highly resistant to false smut after screening of 98 genotypes while 45 of them were resistant and 26 showed 5-70% infection. A screening experiment of 11 rice varieties by Jin *et al* (2005) for resistance to false smut found rice variety 9522 as susceptible while Crystal3 and Dongfan1 was found to be resistant and moderately resistant to false smut respectively under field conditions. Moreover, the severity of the false smut was more at the centre of field than at the edges.

The inheritance of resistance to rice against false smut was studied in F_{10} using 157 recombinant inbred lines (RILs) derived from the cross between resistant cultivar IR 28 (*Oryza sativa* supsp. *indica*) and susceptible cultivar, Daguando (*Oryza sativa* supsp. *japonica*). They used mixed major genes and polygene inheritance approach for studying the inheritance. They found that the inheritance of resistance was controlled by mixed two major genes with equal effects and polygenes with minor effect in which two major genes showed 76.67% of the heritability while the polygenes recorded 22.86% heritability. The results showed that both the effects of major and polygene should be considered in breeding for false smut resistance in rice (Li *et al* 2008).

Chen *et al* (2009) used artificial inoculation of *U. virens* for studying the virulence differentiation of the pathogen and pattern of resistance against false smut in 198 rice varieties. They found that among 198, 44 varieties show immune responses whereas varieties such as Nanjing 17113.34, Yandao 1201 were highly resistant; Changyou 008, Huai 66, Huai 68.29 were resistant and Xiangyou 953.37, Cunsanli varieties were moderately resistant. The plants showing moderately susceptible response includes A2, Mangxiaobaiwan-31 while Zaochadao-15, Xiangzinuo varieties were found to be susceptible and Yanyou 1120.8, Tianfeng you 559 varieties were highly susceptible to false smut of rice.

The significant differences in resistance of rice hybrids against false smut of rice has also been reported in China by Haung *et al* (2010). The study included screening of rice hybrids from different regions of China. Among 122 rice hybrids, most of them were found to be susceptible while some showed high resistance as well as susceptibility to false smut. A method used by Ashizawa *et al* (2011) for evaluating false smut resistance under greenhouse conditions in 18 varieties includes cultivation of the main culm of the rice plant and standardization of conidial spore suspension for inoculation by boot injection. The study also evaluated these varieties under field conditions in 2007 and 2008 for comparisons however, the greenhouse method was found better and more reproducible. This was because the false smut balls were not developed enough on plants to be evaluated owing to low temperatures at the heading stage of rice. The disease severity was measured from the number of smut balls produced per panicle of the plant. The study revealed that the commercial varieties were found to be resistant and varieties showing resistance to blast disease were also mostly

resistant to false smut while the high-yielding forage varieties were highly susceptible. The study concluded that the blast resistant varieties could be used for breeding against false smut of rice while the forage varieties are at high risk of disease.

Mohiddin *et al* (2012) found HR119 variety recording lowest disease incidence after screening of four rice genotypes. Haiyong (2012) used spore suspension of 2008-33-1 *U. virens* isolate for evaluation of two inoculation methods of false smut in rice variety Gangxing 707. They found that crop inoculated at boot stage produced highest false smut incidence (50.43%) with conidial injection than spraying suspension of conidia (34.75%). They screened 20 rice cultivars under artificial conditions. Among these, Fuyou102 and Tyou272 were found to be moderately resistant to false smut with disease incidence of 15.40% and 23.10% respectively. Out of 20 screened cultivars, 11 were highly susceptible while 7 were susceptible to the disease. Later on, they tested the eight *U. virens* isolates on 6 rice varieties using the above successful inoculation technique. The results showed that only Fengyouxiangzhan variety was found to be highly susceptible to *U. virens* isolate 2007-79-1 and Nongfengyou 256 rice variety was moderately resistant while all the other varieties tested were either susceptible or moderately susceptible. The rice varieties Jixiangyou 830, Gangyou 827 and Fengyouxiangzhan were among the most susceptible varieties (Haiyong *et al* 2015).

Lore *et al* (2013) found quantitative variation in susceptibility level among rice genotypes against false smut of rice. After screening of 25 rice hybrids and 11 inbred lines, it was found that two hybrids NPH 909 and NPH 369 showed higher disease intensity while the hybrid cultivars, PR113 and PR114 exhibit lowest level of disease intensity. The genotypes used in the experiment were grouped into five groups from the hierarchical cluster analysis using Mahalanobis distance. The first group exhibits highest average of per cent infected spikelets (18.13 %), number of smut balls produced per panicle (22.00) and mean disease score (8.33). this was followed by the second, third and the fourth group based on the above disease parameters. The fifth group consist of lowest mean values of per cent infected spikelets (0.51%), number of smut balls produced per panicle (0.67) and mean disease score (1.33).

Yan *et al* (2014) screened various rice varieties including the newly registered commercial rice hybrids (Xindu and Qianglai) against false smut of rice. Among all tested varieties, Gangyou 725 showed higher disease severity in the form of infected panicles than Zhongyou 44. Kaur *et al* (2015) evaluated 125 rice genotypes, consisting of hybrids and 18 inbred lines from National Hybrid Screening Nursery, by spray inoculation of spore suspension on panicles. The genotypes were divided into four groups on the basis of average values of disease variables for scoring of false smut disease as done by Lore *et al* (2013). The study found one inbred line and nine hybrids resistant to false smut disease. The early and

medium duration rice genotypes were also evaluated by Singh and Sunder (2015) for their response to false smut. Among the total 123 rice genotypes screened, 20 genotypes showed resistant response while 91 genotypes were moderately resistant to false smut.

Rani *et al* (2016) evaluated 31 rice germplasm lines for identification of resistance to false smut. Among the tested lines, 10 lines were found highly resistant against the disease based on the disease variables. In succession to this, evaluation of five germplasm lines in 2014 showed that all the tested lines were found to be completely free from the false smut disease. Raji *et al* (2016) screened twenty rice varieties for resistance to false smut under field conditions through natural selection. The varieties were divided into four groups based on the infected florets per panicle and corresponding reaction according to standard evaluation system. The study found seven rice varieties Ptb7, Ptb23, Ptb24, Ptb32, Ptb36, Ptb42 and Ptb46 resistant to false smut under field conditions. Fu *et al* (2016) evaluated 843 rice accessions against false smut disease and found that 179 rice accessions showed resistance to disease. Furthermore, 36 accessions were evaluated again at two locations against false smut and found three accessions showing no disease symptoms in all the trials.

Banasode and Hosagoudar (2021) screened 102 rice genotypes under field conditions based on natural selection to identify resistant sources against false smut in Karnataka. They found 11 genotypes *viz.*, (IET 24956, IET 25530, IET 26273, IET 26218, IET 26275, IET 25798, IET 24995, IET 25523, Varshadhan, IET 27274 and IET 27277) were highly resistant and IR-64 was moderately resistant to disease on the basis of per cent infected panicles of rice. On the other hand, 53 genotypes were moderately susceptible and 34 were susceptible while three genotypes *viz.*, IET 24518, IET 25191 and IET 26219 were highly susceptible to the disease. The resistant and susceptible check used were IR-64 and Tunga, respectively. The researchers concluded that the high degree of variability in the false smut resistance among rice cultivars might be attributed to differences in genetic makeup of the cultivars as well as environmental factors that plays a major role in host- pathogen interactions.

Hiremath *et al* (2021) screened 125 rice accessions from global rice diversity panel for identification of potential donors for false smut resistance. They screened the accessions under natural hotspot for three consecutive years as well as under artificial inoculation conditions in the fourth year using spray inoculation technique. The disease parameters such as infected panicles per plant, number of smut balls per panicle and disease score was used to access the resistance level of accessions. The commercial variety PR116 and an advanced breeding line, GSR123, were used as susceptible checks as they are highly susceptible to false smut. Out of 125 germplasm lines, 25 lines were highly resistant in all the four years under natural as well as artificial conditions whereas 31 lines were moderately resistant to false smut.

2.11 Quantitative trait loci for resistance to false smut in rice

Breeding and utilization of resistant cultivar is the most effective and economical way to control false smut disease which ensure the high yield of crop. Till date no high level of resistance source has been found in rice germplasms. The quantitative resistance conferred by quantitative trait loci is a valuable resource for improvement of the resistance of rice against different diseases (Kou and Wang 2011). The crossing and backcrossing procedure has been used widely for transfer of resistance and other important traits from wild species to cultivated varieties. The qualitative gene resistance is governed by single gene and is pathogen race specific but no R gene has been identified so far against some serious crop diseases like false smut of rice (Kou *et al* 2010).

Marker assisted selection approach has been used successfully to develop varieties resistant to various diseases caused by plant pathogens such as *U. virens* (Xu *et al* 2002, Li *et al* 2011). Among all the markers, the most commonly used markers for mapping and tagging of genes in rice are micro-satellites or SSRs due to abundance of these markers. Till date, 28 QTLs have been mapped using bi-parental populations from crosses of resistant and susceptible cultivars (Neelam *et al* 2021).

Using near isogenic introgression line for disease resistance two QTLs were mapped (Xu *et al* 2002) using simple sequence repeat (SSR) markers. In a study by Li *et al* (2008) regarding the inheritance to rice false smut, a population of 157 recombinant inbred lines which were derived from the cross between resistant cultivar IR 28 (*Oryza sativa* subsp indica) and susceptible landrace Daguandae (*Oryza sativa* subsp japonica) was analysed using the major genes and polygenes inheritance model.

Li *et al* (2008) mapped two major genes controlling rice false smut with polygene mixed model. They also studied the inheritance of rice false smut with the help of a population derived from the cross between resistant cultivar IR28 (*O. sativa* subsp *indica*) and susceptible landrace Daguandae (*O. sativa* subsp *japonica*). The cross resulted in 157 recombinant inbred lines, which were further analysed using the major genes and polygenes inheritance model. Li *et al* (2011) obtained quantitative trait loci conferring resistance to rice false smut from a recombinant inbred line population with 157 lines. The disease rate index of two parents and 157 RILs caused by rice false smut were scored and the QTLs for false smut resistance were detected by the QTL Cartographer software. Further, eight QTL's (qFsr1, qFsr2, qFsr4, qFsr8, qFsr10a, qFsr10b, qFsr11 and qFsr12) controlling false smut resistance were detected on chromosomes 1, 2, 4, 8, 10, 11 and 12 respectively, with the phenotypic variation of 9.8 per cent to 22.5 per cent. Similarly, five QTLs were detected in Nanjing and Yangzhou respectively (Li *et al* 2014).

Quantitative trait loci were also found from 213 introgression lines obtained from a cross between Teqing (recipient) and Lemont (donor) using natural infection in China. The

study was conducted with the aim of breeding rice varieties possessing high level of field resistance to the false smut disease. Out of this, ten QTL that affect the incidence of disease were detected and used for mapping rice chromosomes number 2, 3, 4, 6, 8, 10, 11 and 12. The presence of alleles of Lemont at all quantitative resistant loci (QRL) were responsible for increased resistance to false smut. Across the two testing sites among all the QRL, four QRL (qFSR-6-7, qFSR-10-5, qFSR-10-2 and qFSR-11-2) had relatively larger and consistent effects. This study was helpful in identifying promising resistant introgression lines exhibiting multiple QRL linked with their markers through marker-assisted selection (Zhou *et al* 2014).

A study suggested that the durable and broad spectrum disease resistance for sheath blight of rice might be associated with chitinase gene cluster (Channamallikarjuna *et al* 2010). Nine chitinase genes were found to be highly induced after the infection by *U. virens* (Deepan *et al* 2017). Apart from that, the chitinase gene cluster region were also described closer to the QTL conferring resistance to false smut (Han *et al* 2015).

Han *et al* (2020) mapped five QTLs associated with resistance to false smut of rice on chromosomes 2, 4, 8, and 11. The used 200 recombinant inbred lines in F_7 as mapping population derived from a cross between MR183-2 (resistant cultivar) and 08R2394 (highly susceptible cultivar) by single seed descent method. They found total of 179 polymorphic SSR markers for constructing genetic linkage maps. Among all QTLs, a novel QTL *qFSr8-1* reported on chromosome 8 represents major QTL accounting for the largest phenotypic variance and several candidate genes in this region are predicted to be involved in disease resistance to false smut. Another QTL for heading date, *qHd8–1* was also mapped to the same region as that of QTL *qFsr8–1* controlling false smut resistance. This shows that both the QTLs are tightly linked to each other.

Recently, single major locus, *FSR1* (*false smut resistance 1*) was fine mapped to 220-kb on long arm of chromosome 1. The study used an F_2 population derived from the cross between Nanjing11 (false smut resistant) and CG3 (false smut susceptible). A novel resistance gene *FSR1* encoding a *DNA methylase 2* (LOC_Os01g42630) was identified as a putative candidate gene in the mapped region of Nanjing11 for false smut resistance based on sequence variation and transcriptional responses to *U. virens* (Qiu *et al* 2020).

Apart from bi-parental populations, genome-wide association studies (GWAS) have been promisingly used for identification of resistant QTLs against false smut of rice using diverse rice accessions. GWAS take advantage of historical recombination events in diverse sources to resolve QTL to the sequence level (Zhu *et al* 2008). It finds the association between a large number of markers and trait of interest here, false smut resistance, to understand the variation due to complex traits governed by many genes. The QTL study does not cover the whole genome for identifying the phenotypic variation. QTL mapping for false

smut resistance are based on F_2 or other segregating populations, the identified QTLs could not be evaluated repeatedly and are subjected to higher experimental errors. This is a major drawback in QTLs effective evaluation and their further use in breeding program against *U. virens* (Andargie *et al* 2018).

In GWAS analysis of rice false smut resistance, a total of 315 global diversity panel rice accessions were screened against *U. virens.* The genotyping of 271,360 SNPs yielded 58 significant SNPs which were distributed on the rice whole genome except chromosome 7. Three SNPs were found to be associated with three rice false smut resistance related traits *viz.,* disease plant percentage (DPP), disease panicles per disease plant (DPPDP) and disease smut balls per disease plant (DBPDP). These three new loci for rice false smut resistance were identified repeatedly in three traits during the 2 years of study. The results imply that these three associated SNPs represents major locus which is stable against false smut over the years. Moreover, these three SNPs (chr01_8601035, chr01_8738751, and chr01_9107381) are found clustered together thus, can be used in gene pyramiding in the breeding programs for resistance to false smut. After gene annotation, they found that only one disease resistance protein (RGA2), is located 12.3 kb far away from one of the repeatedly detected significant SNP (chr01_8738751). It was encoding NBS-LRR protein which is known to be involved in disease resistance. This implies that RGA2 might be contributing to false smut resistance in rice (Long *et al* 2020).

Hiremath *et al* (2021) screened 125 rice accessions from global rice diversity panel for identification of false smut resistance in rice. They screened the accessions under natural hotspot for three consecutive years as well as under artificial inoculation conditions in the fourth year using spray inoculation technique. The disease parameters such as infected panicles per plant, number of smut balls per panicle and disease score was used to access the resistance level of accessions. The commercial variety PR116 and an advanced breeding line, GSR123, were used as susceptible checks as they are highly susceptible to false smut. GWAS for the above false smut related traits revealed significant associations on chromosome 2,3,6,9 and 11 for infected panicles per plant (IPP). Moreover, significant associations for number of smut balls per panicle (NSBP) on chromosomes 3 and 8, while for disease score (DS) on chromosomes 3,4 and 11 of rice.

Neelam *et al* (2021) used resistant breeding line RYT2668 (resistant) and Punjab Rice 116 (susceptible variety) for developing mapping population of 250 recombinant inbred lines (RILs, F_9) to identify false smut resistant QTLs using high density single nucleotide polymorphism (SNP) markers. The mapping population was evaluated for false smut resistance in 2013, 2015 and 2016 under field conditions. They mapped seven QTLs on rice chromosomes 2,4,5,7 and 9 using 2326 SNP markers. Out of seven, two QTLs, *qRFSr5.3* and

qRFSr7.1a were found to be associated with infected panicle per plant, QTL *qRFsr9.1* with total number of smut balls per panicle and four QTLs, *qRFSr2.2*, *qRFSr4.3*, *qRFSr5.4*, and *qRFSr7.1b*, with disease score. All the QTLs reported were novel except *qRFSr4.3* (Li *et al* 2011). Among all, QTL *qRFSr9.1*, is a novel QTL found on chromosome 9 exhibiting large phenotypic effect and QTL *qRFSr7.1b* to be mapped on chromosome 7 for the first time. Four disease resistance proteins with NBS-LRR domain were found as putative candidate genes associated within this QTL. The research will be helpful in identification of disease resistant genes/QTLs to false smut present on rice chromosomes.

2.12 Management of false smut of rice

Rice false smut caused by *U. virens* is an increasing threat to grain yield due to its severe epidemics and causes both quantitative and qualitative losses in rice production. Host plant resistance and breeding for false smut resistance is the most effective and economical method of management of false smut. We have limited resistant sources available against this disease (Raji *et al* 2016, Hiremath *et al* 2021). The major drawbacks in identification of resistant sources against this disease were due to its minor importance in the past decades, lack of standardized inoculation technique and difficulty in obtaining axenic culture of the pathogen for artificial inoculation. The inheritance of RFS resistance is not well understood, breeding for resistant rice is hampered. Late maturing rice varieties with high grain density are more vulnerable to this disease (Wang *et al* 2019)

2.12.1 Cultural practices for management of false smut

Cultural practices aim to reduce incidence of false smut by manipulating various rice cultivation practices. The occurrence of false smut of rice is related with high humidity (>90%) and rainfall during flowering under highly fertile soil conditions (Ahonshi *et al* 2000). Early maturing rice varieties show less incidence of false smut than late maturing rice varieties. In a two-year survey conducted by Atia (2004) on yield losses of rice, it was found that early maturing rice varieties showed less disease incidence of false smut as compared to late maturing rice varieties. Moreover, the disease incidence was more in case of soils rich in nitrogen fertilization, sandy or loose soils and late transplanting of rice. The early maturing rice genotypes escape disease because relative humidity and temperature are higher later in the season which is highly conducive for the development of the pathogen (Nessa *et al* 2015).

Therefore, early planting of rice should be encouraged as well as excess application of nitrogenous fertilizer should be avoided and adopt fertilizer ratio approach for judicious use of fertilizers. Besides rice, the alternative hosts of *U. virens* includes several weed species suggesting the role of weeds in the growth of pathogen and disease cycle (Atia 2004). Therefore, regular cleaning of field ridges and irrigation channels for destruction of

alternative hosts and reduction of primary inoculum of pathogen (Fan *et al* 2014). Other practices include specific crop rotation, furrow irrigation, conservation tillage, suitable plant and use of disease free healthy seeds are also recommended (Brooks *et al* 2009, Brooks *et al* 2010, Brooks *et al* 2011). Therefore, in areas with high false smut incidence integrated management approaches are recommended (Sun *et al* 2020).

2.12.2 Chemical control

Chemical control through the application of chemical fungicide is considered non-economical and non-environment-eco-friendly but rice false control largely relies on use of fungicides at specific stages of rice crop. Therefore, fungicides with low toxicity effects, high efficiency and low residue are used widely to control false smut of rice (Hu *et al* 2018). Among various fungicides tested so far, propiconazole, azoxystrobin, Wenquning (*Bacillus subtilis* suspension in validamycin), cuproxat SC, copper oxychloride, difenoconazole, simeconazole, tebuconazole and hexaconazole, are highly effective to reduce incidence of false smut in rice (Ahonsi M O and Adeoti 2003, Bagga and Kaur 2006, Zhou and Wang 2011, Chen *et al* 2013, Liang *et al* 2014, Muniraju *et al* 2017, Zhou *et al* 2019). Moreover, Mixture of fungicides has also proven to be highly effective against *U. virens* infection such as combination of azoxystrobin and difenconazole leads to 94% reduction in disease incidence than using azoxystrobin and difenconazole solely (Muniraju *et al* 2017).

Apart from the chemistry of fungicides, the timing of fungicide application to rice crop is also very crucial for the effective management of the disease. Generally, the pathogen infects the rice before anthesis and at late booting stage *via* a small gap between lemma and palea (Ashizawa *et al* 2012, Tang *et al* 2013). Therefore, the fungicides should be applied at booting stage of crop to prevent the entry of pathogen and its further build-up in plant. Application of fungicides after panicle heading should be prevented, as the pathogen infects rice flowers at late booting stage and already successfully colonizes the inner floral organs after heading. The false smut of rice was effectively controlled by simeconazole fungicide when applied three weeks before heading of the rice crop (Tsuda *et al* 2006, Liang *et al* 2014).

2.12.3 Genetic resistance

The different cultivars grown under the same location and environmental conditions with similar agronomic practices vary in disease incidence and severity to false smut of rice. This may be attributed to the genetic background of variety having some potential sources of resistance to the pathogen (Ou 1985).

Lore *et al* (2013) found quantitative variation in susceptibility level among rice genotypes against false smut of rice. After screening of 25 rice hybrids and 11 inbred lines, it

was found that two hybrids NPH 909 and NPH 369 showed higher disease intensity while the hybrid cultivars, PR113 and PR114 exhibit lowest level of disease intensity.

Yan *et al* (2014) screened various rice varieties including the newly registered commercial rice hybrids (Xindu and Qianglai) against false smut of rice. Among all tested varieties, Gangyou 725 showed higher disease severity in the form of infected panicles than Zhongyou 44. Kaur *et al* (2015) evaluated 125 rice genotypes, consisting of hybrids and 18 inbred lines from National Hybrid Screening Nursery, by spray inoculation of spore suspension on panicles. The study found one inbred line and nine hybrids resistant to false smut disease. The early and medium duration rice genotypes were also evaluated by Singh and Sunder (2015) for their response to false smut. Among the total 123 rice genotypes screened, 20 genotypes showed resistant response while 91 genotypes were moderately resistant to false smut.

The screening of 843 rice accessions for a period of three years was done by Huang *et al* (2016). They found 36 accessions with no incidence of false smut disease. The study found several resistant accessions through polymorphism analysis which could be used for to construct gene mapping populations after hybridization with Pujiang 6, a susceptible accession identified in this study.

Banasode and Hosagoudar (2021) screened 102 rice genotypes under field conditions based on natural selection to identify resistant sources against false smut in Karnataka. They found 11 genotypes *viz.*, (IET 24956, IET 25530, IET 26273, IET 26218, IET 26275, IET 25798, IET 24995, IET 25523, Varshadhan, IET 27274 and IET 27277) were highly resistant and IR-64 was moderately resistant to disease.

The quantitative resistance conferred by quantitative trait loci is a valuable resource for improvement of the resistance of rice against different diseases (Kou and Wang 2011). Marker assisted selection approach has been used successfully to develop varieties resistant to various diseases caused by plant pathogens such as *U. virens* (Xu *et al* 2002, Li *et al* 2011). Among all the markers, the most commonly used markers for mapping and tagging of genes in rice are micro-satellites or SSRs due to abundance of these markers. Till date, 28 QTLs have been mapped using bi-parental populations from crosses of resistant and susceptible cultivars (Neelam *et al* 2021).

Li *et al* (2011) and Li *et al* (2014) detected eight QTLs (qFsr1, qFsr2, qFsr4, qFsr8, qFsr10a, qFsr10b, qFsr11 and qFsr12) on chromosomes 1, 2, 4, 8, 10, 11 and 12 controlling false smut resistance. Four quantitative resistant loci (qFSR-6-7, qFSR-10-5, qFSR-10-2 and qFSR-11-2) were identified having relatively larger and consistent effects after testing at two locations (Zhou *et al* 2014). Han *et al* (2020) mapped five QTLs associated with resistance to false smut of rice on chromosomes 2, 4, 8, and 11. Another QTL for heading date was also

mapped to the same region as that of QTL *qFsr8–1* controlling false smut resistance. Recently, single major locus, *FSR1* (*false smut resistance 1*) was fine mapped to 220-kb on long arm of chromosome 1 (Qiu *et al* 2020).

In GWAS analysis of rice false smut resistance, three SNPs (chr01_8601035, chr01_8738751, and chr01_9107381) clustered together were found to be associated with three rice false smut resistance related traits *viz.*, disease plant percentage (DPP), disease panicles per disease plant (DPPDP) and disease smut balls per disease plant (DBPDP) during the 2 years of study. After gene annotation, they found that only one disease resistance protein (RGA2), is located 12.3 kb far away from one of the repeatedly detected significant SNP (chr01_8738751) and was encoding NBS-LRR protein which is known to be involved in disease resistance. This implies that RGA2 might be contributing to false smut resistance in rice (Long *et al* 2020).

Hiremath *et al* (2021) screened 125 rice accessions from global rice diversity panel for identification of false smut resistance in rice. GWAS for the above false smut related traits revealed significant associations on chromosome 2,3,6,9 and 11 for infected panicles per plant (IPP). Moreover, significant associations for number of smut balls per panicle (NSBP) on chromosomes 3 and 8, while for disease score (DS) on chromosomes 3,4 and 11 of rice.

Neelam *et al* (2021) identified false smut resistant QTLs using high density single nucleotide polymorphism (SNP) markers. They mapped seven QTLs on rice chromosomes 2,4,5,7 and 9 using 2326 SNP markers. All the QTLs reported were novel except *qRFSr4.3* (Li *et al* 2011). Among all, QTL *qRFSr9.1*, is a novel QTL found on chromosome 9 exhibiting large phenotypic effect and QTL *qRFSr7.1b* to be mapped on chromosome 7 for the first time. Four disease resistance proteins with NBS-LRR domain were found as putative candidate genes associated within this QTL.

CHAPTER III

MATERIALS AND METHODS

The study was undertaken to study the role of primary inoculum in carryover of the disease and to identify and map quantitative trait loci governing resistance to false smut in rice. The present studies will generate information on mode of survival of the pathogen and identification of QTLs for resistance to false smut which can be used in development of resistance varieties. The experiments pertaining to the study was carried out at Department of Plant Pathology (PAU) and Experimental area, Department of Plant Breeding and Genetics, PAU Ludhiana. The materials and methods have been described under the following sub-headings.

3.1 Isolation, identification and purification of the causal organism

3.2 Preparation of inoculum and inoculation methods

3.3 Preparation and sampling of plant material (PR116) for detection of primary inoculum of *U. virens*

3.4 DNA extraction from axenic culture of *U. virens*

3.5 Polymerase chain reaction for nested PCR protocol

3.6 Visualization of PCR product

3.7 Screening of rice germplasm accessions for identification of potential donors imparting resistance to false smut under field conditions

3.8 Quantitative trait loci mapping for resistance to false in rice

3.1 Isolation, identification and purification of the causal organism

3.1.1 Preparation of media

The media used for isolation and multiplication of *Ustilaginoidea virens* was potato sucrose agar (PSA) with the following composition (Table 3.1).

Table 3.1: Composition of the Potato Sucrose Agar (PSA) medium

Peeled potato slices	250 g
Sucrose	20 g
Agar	20 g
Distilled water	1000 ml

The peeled and sliced potato (250g) was boiled in 500 ml of distilled water for 20-30 minutes. The potato extract was then filtered using muslin cloth and sucrose (20g) was added to the filtered extract. The filtrate was mixed gently with the help of glass rod until the

sucrose dissolves properly in the filtrate. Thereafter, the final volume was prepared to 1000 ml by adding distilled water. The agar (20g) was added to the medium slowly after the media gets cool down to prevent formation of lumps in the medium. About 250 ml of media was poured into 500 ml flasks each and plugged with non-absorbent cotton plug. The flasks containing media was autoclaved at temperature 121°C, 15 lbs psi pressure for about 20 minutes. The potato sucrose broth (PSB) was prepared in the same manner except the addition of agar for mass multiplication of the pathogen. The test tubes containing the PSA medium @ 5ml per tube were autoclaved at temperature 121°C, 15 lbs psi pressure for about 20 minutes. The autoclaved test tubes were allowed to solidify in slanting position in order to prepare slants which are further used for maintenance of pure culture of pathogen.

3.1.2 Isolation and purification of *U. virens*

The smut balls were collected from infected rice panicles and stored in the refrigerator at 4°C. Isolations were made from the samples collected from previous season crop (Plate 2). The surface sterilization was done with 0.1 per cent mercuric chloride solution in an autoclaved test tube for one minute. Subsequently, autoclaved sterile distilled water in test tubes was used for giving three washings to the sterilized smut balls by shaking the tubes containing smut balls constantly. This step will remove the residues of mercuric chloride in the smut balls after surface sterilization. Then, the sterilized smut balls were dried using the filter paper placed in the Petri-dish. With the help of sterilized inoculating needle, the mass of chlamydospores from the smut balls were streaked on to the Petri-dishes containing potato sucrose agar (PSA) in laminar flow aspetically. The inoculated Petri-plates after applying parafilm were incubated in BOD incubator at 25±2°C for 8-10 days for obtaining pure culture of the fungus. The incubated plates should be checked and examined periodically for the growth of the fungal mycelium according to its morphological characteristics.

After the appearance of fluffy white cottony growth of the mycelium (Plate 2), it was transferred to fresh PSA Petri-dishes with the help of sterilized inoculating needle in laminar flow for obtaining axenic culture of *U. virens.* After five days of incubation under same above conditions, a single, isolated colony of the fungus was picked up with the help of sterilized inoculating needle and transferred to test tubes containing PSA (slants) for maintenance of pure culture. The pure fungus colony growth was confirmed further by observing under microscope for mass of conidia or mycelial characteristics of the fungus under 40X (Plate 3). The slants were observed periodically for the growth of pure fungus (Plate 2). These slants were used further for mass multiplication of the fungus in PSB and/or stored in refrigerator at 4°C for storage and future use.

3.1.3 Mass multiplication of *Ustilaginoidea virens*

The pure culture of the fungus in slants was used for inoculating PSB in 500 ml flasks

Plate 2: Pure culture of *Ustilaginoidea virens* (*Villosiclava virens*) on PSA medium. (a) Infected smut balls from previous season, (b) Conidial culture, (c,d) Smut balls containing chlamydospores, (e) Conidial culture on slants, (f) Conidial culture on PSB medium and (g) Mass multiplication of *U. virens.*

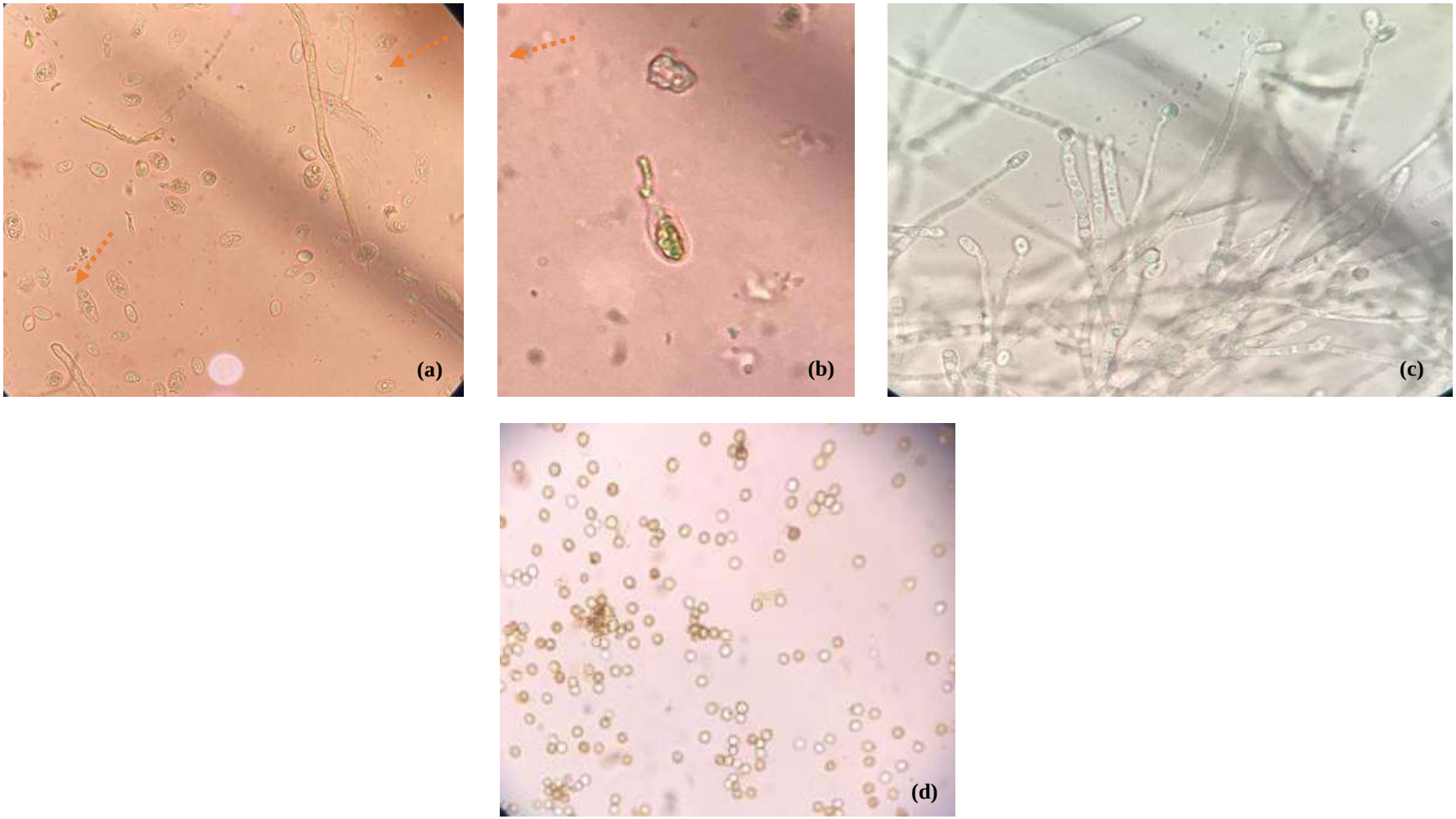


Plate 3: Microscopic visualization of *Ustilaginoidea virens* (*Villosiclava virens*) (a) Conidia under 40X (b) Germinating conidia under 100X (c) Conidia borne on conidiophores under 100X (d) Chlamydospores under 40X.

for mass multiplication of *U. virens* and its further use in inoculation experiments (Plate 2). The flasks were incubated at 25±2°C for 8-10 days on shaker at 125 rpm. For DNA isolation f *U. virens*, the mycelial mat formed in PSB after 10-15 days of constant shaking was used.

3.2 Preparation of inoculum and inoculation methods

The inocula prepared for the artificial inoculation methods were of two types. The chlamydospores harvested from the freshly collected smut balls from the diseased rice panicles were used for the preparation of chlamydospore suspension. For preparation of conidial suspension, the conidia are harvested from the mycelium grown under *in-vitro*. The haemocytometer was used for adjusting the concentration of both suspensions to 7.5×10^5 chlamydospores/ conidia ml^{-1} (Ashizawa *et al* 2011). The germplasm lines of rice grown under field conditions were inoculated with chlamydospore/conidial suspension at boot stage of crop by spraying using an aerosol spray bottle until the glumes gets wet and suspension drop down from the entire panicle (Plate 4).

On the other hand, the mycelial mat of the fungus which was produced in the flask (section 3.1.3) was filtered and conidia were harvested as well as suspended in sterile distilled water for inoculation. The concentration of conidial suspension was adjusted to 2×10^5 conidia ml^{-1} with the help of haemocytometer (Ladhalakshmi *et al* 2012). The selected germplasm lines to be evaluated under artificial conditions were inoculated at boot stage by injecting 1-2ml of conidial suspension into the leaf sheath from the base until it overflowed with the help of hypodermic sterile syringe (Plate 4). Generally, the inoculations were done during the evening hours to prevent desiccation of fungal spores in the morning and afternoon hours. The plants injected with sterile water served as control. Proper tagging of the inoculated plant as well as panicle is done marked with date and type of inoculation method. After inoculation, perforated sprays with spray bottle is done to maintain relative humidity (95%) for at least one week. The plants were incubated at temperature 25°C and 95% relative humidity for two days thereafter, the plants were maintained under greenhouse at 25-28°C for a week.

3.3 Preparation and sampling of plant material (PR116) for detection of primary inoculum of *U. virens*

3.3.1 Determination of role of seed in primary source of inoculum of false smut disease in rice

The susceptible check PR116 was used in this study. The healthy and disease free seeds of rice cultivar PR116 was collected. Nested-PCR primers US2-5/ US4-3 was used to detect and quantify *U. virens* from seeds (Zhou *et al* 2003). Similarly, the infected seed was collected and inoculated by dipping them in freshly prepared inoculum of *U. virens* (section

3.1.3). Two treatments were maintained one with healthy seed (T1) and the other with infected seed (T2, Table 3.2). The healthy and infected seeds so selected was grown in earthen pots containing sterilized soil, obtained by autoclaving. For each treatment, four replicates with 5 pots in each replicate were maintained in a completely randomized block design (CRD). In each pot, three rice plants were maintained throughout the period of experimentation (Plate 5). All the agronomic and cultural operations were done as per recommendations of Punjab Agricultural University, Ludhiana for rice (Anonymous, 2019). A total of 120 plants was raised. Frequent water sprays to maintain optimum moisture conditions. The plants were covered with bags before the panicle emergence stage to prevent the air borne inoculum from infecting the plant. Five seedlings were sampled per replicate at 10 days interval after emergence from the soil until booting stage and tested for *U. virens* rDNA using the nested-PCR protocol. The seedlings samples were gently removed from the soil and washed under running tap water for 30 seconds. While in tests involving sheath of booted panicles, after washing the sheath is separated from the main culm of plant aseptically. The samples are then used for DNA extraction procedures. The disease assessment and various observations on disease variables were recorded (section 3.7.2, Plate 6).

3.3.2 Determination of role of soil in primary source of inoculum of false smut disease in rice

The sick soil was taken from the plot where the susceptible checks heavily infested with false smut are grown in each successive year. The pure mass culture of *U. virens* (section 3.1.3) was used for mixing it with sick soil. The susceptible check PR116 was used in this study. The healthy seed of PR116 were selected (section 3.3.1) and grown in earthen pots containing infected soil (T3). The earthen pots containing sterilized soil was used as check (T4, Table 3.2). For each treatment, four replicates with 5 pots in each replicate were maintained. In each pot, three rice plants were maintained throughout the period of experimentation. A total of 120 plants was raised (Plate 5). Frequent water sprays to maintain optimum moisture conditions. The plants were covered with bags before the panicle emergence stage to prevent the air borne inoculum from infecting the plant. The sampling was done as mentioned under section 3.3.1. The disease assessment and various observations on disease variables were recorded (section 3.7.2, Plate 6).

3.3.3 Determination of role of air-borne spores in primary source of inoculum of false smut disease in rice

The susceptible check PR116 was used in this study. The healthy and disease free seeds of rice cultivar PR116 was collected. The healthy seeds so selected was grown in earthen pots containing sterilized soil, obtained by autoclaving. For each treatment, four replicates with 5 pots in each replicate were maintained. In each pot, three rice plants were maintained throughout the period of experimentation. A total of 120 plants was raised (Plate

(a)

(b)

Plate 4: Artificial inoculation of *Ustilaginoidea virens:* (a) Conidial injection at boot stage (b) Spray inoculation of conidia/chlamydospore suspension.

Plate 5: Experimental view of pot experiment with different treatments (T1-T6) of PR116.

(a) **(b)**

(c) **(d)** **(e)**

Plate 6: Disease score on 0-9 scale (a) Disease score 1 (b) Disease score 3 (c) Disease score 5 (d) Disease score 7 (e) Disease score 9.

5). Frequent water sprays to maintain optimum moisture conditions. The plants were not covered with bags before the panicle emergence stage to allow the air borne inoculum infecting the plant (T5). The plants covered with bags were used as control (T6, Table 3.2). The sampling was done as in mentioned under section 3.3.1. The disease assessment and various observations on disease variables were recorded (section 3.7.2, Plate 6).

Table 3.2: Different treatments selected for above experiment on role of primary inoculum of *U. virens*

Treatment	Source
T1	Healthy seed
T2	Infected seed
T3	Infected soil
T4	Sterilized soil
T5	Uncovered panicles
T6	Covered panicles

3.3.4 DNA extraction from rice tissues

Five seedlings were sampled per replicate at 10 days' interval after emergence from the soil until booting stage and tested for *U. virens* rDNA using the nested-PCR protocol. The seedlings samples were gently removed from the soil and washed under running tap water for 30 seconds. While in tests involving sheath of booted panicles, after washing the sheath is separated from the main culm of plant aseptically. The samples are then used for DNA extraction procedures.

The total DNA from the rice tissues was extracted by Cetyl trimethyl ammonium bromide (CTAB) method. The pestle and mortar are autoclaved or surface sterilized by 70% ethanol. The liquid nitrogen was used initially for chilling the pestle and mortar. Afterwards, 100 mg (approximately) of rice tissues was crushed in pre-chilled pestle and mortar using liquid nitrogen. The leaves were grinded to powder form and transferred to 2ml Eppendorf tubes. 750 µl of pre-warmed CTAB buffer (65°C, Table 3.3) was added to the powdered tissue and the samples were suspended in buffer by inverting the Eppendorf tubes up and down to mix the contents properly. The tubes are incubated in pre-heated water bath at 65°C for 45 minutes and the tubes are inverted again after sometime for complete mixing of the contents. After incubation, the tubes were vortexed at 13,000 rpm for 15 minutes and 800 µl mixture of chloroform: isomyl alcohol (24:1) prepared in equal volume was added to lyse the samples. The tubes were placed on rotary shaker for 30 minutes and then the samples were

centrifuge at 13,000 rpm for 15 minutes at room temperature. After centrifugation, there was formation of three phases. The upper aqueous phase containing the DNA was transferred into a clean and sterile 1.5 ml Eppendorf tube. The above chloroform: isomyl alcohol step was repeated to extract the entire DNA. An equal volume of pre-chilled isopropanol was added to the pooled supernatant and the tubes were incubated for an hour or overnight at -20°C for precipitating the DNA. The DNA was precipitated by centrifugation at 13,000 rpm for 15 minutes at 4°C. Following centrifugation, the DNA was pelleted at the bottom of the tube and the supernatant containing isopropanol was discarded carefully without disturbing the DNA pellet. The DNA pellet was washed twice with 500 µl chilled 70% ethanol to remove residual salts and centrifuged at 13,000 rpm for 5 minutes at 4°C. The pellet was air-dried completely from ethanol by turning down the centrifuge tubes on the blotter paper. After drying, 100 µl of 1X TE buffer (Tris EDTA buffer- 10 Mm Tris HCL, 1 mM EDTA, pH 8.0) was added for dissolving the DNA pellet by flicking the tube. The tubes were stored at 4°C overnight and after DNA quantification they were stored at -20°C for further analysis. The final concentration of DNA was checked by 0.8% agarose gel electrophoresis or through spectrophotometer (Sambrook *et al* 1989).

3.3.5 Assessment of quality and quantity of DNA

Quality and quantity of DNA was checked by using Tecan 2000 Nanoquant plate reader. The blanking was done by using 2 µl of 1X TE buffer. The readings were taken by dispensing 2µl of individual DNA samples from different isolates. The quality of DNA was checked at the ratio of 260:280. The reading was taken thrice and average of three reading was used to make dilution for further analysis. The DNA of all the samples was diluted to 25 ng/µl by adding nuclease free water and stored at -20ºC. Alternately, DNA quality was also checked on Agarose gel (0.8%). A single sharp band of DNA signified high quality of DNA.

Table 3.3: Composition of CTAB buffer (pH-8.0)

CTAB	2.0 g
1M Tris (pH-8.0)	10.0 ml
0.5M EDTA (pH-8.0)	4.0 ml
5M NaCl	28.0 ml
Distilled water	40.0 ml
PVP 40 Mw 40,000	1 g
β-Mercaptaethanol	50 µl
Final volume	100 ml

3.4 DNA extraction from axenic culture of *U. virens*

The axenic mass culture of plant pathogenic fungi *U. virens* obtained from section 3.1.3 were used. A pure culture of *U. virens* mycelia was inoculated in PDB and incubated at 25°C for 10-15 days in a rotary incubator shaker at 125 rpm. The mycelial mat was harvested by filtration with help of sterile funnel and dried under sterile conditions with 9.0 cm Whatman filter paper (No. 4). The dried mycelium is wrapped completely in multiple layers of aluminium foil. Extraction of total DNA from the culture mycelium was done by CTAB method (Table 3.3). Cetyl trimethyl ammonium bromide (CTAB) method was adopted to extract the total DNA from the mycelium of *U. virens*. The pestle and mortar are autoclaved or surface sterilized by 70% ethanol. The liquid nitrogen was used initially for chilling the pestle and mortar. Afterwards, dried mycelium wrapped in aluminium foil was crushed in pre-chilled pestle and mortar using liquid nitrogen. The mycelial were grinded to powder form and transferred to 2ml Eppendorf tubes from aluminium foil. Thereafter, the same procedure of DNA extraction was followed as given in section 3.3.4. The tubes with DNA suspended in TE buffer, were stored at 4°C overnight and after DNA quantification they were stored at -20°C for further analysis. The final concentration of DNA was checked by 0.8% agarose gel electrophoresis or through spectrophotometer (Sambrook *et al* 1989).

3.5 Polymerase chain reaction for nested PCR protocol

The specific internal transcribed spacer (ITS) primers were used to confirm *U. virens* (Zhou *et al* 2003). The ITS primer pair (ITS4 and ITS5, Table 3.4) were used for first round of PCR amplification of pathogen DNA. The polymerase chain reaction (PCR) mixture (25 µl) was prepared (Table 3.5). The PCR plate was covered with thermoseal to prevent evaporation of PCR product. The amplification was carried out in 96 well PCR plate with the cycler program (Table 3.6) in Eppendorf Master Cycler ProS. For nested PCR, 1 µl of the product from the first round of PCR with ITS primer pair was used as the template for second round of amplification with nested PCR primers (Table 3.7) using the nested PCR protocol (Table 3.8). The nested PCR primers (Table 3.7) are *U. virens*-specific internal transcribed spacer (ITS) primer.

Table 3.4: Universal ITS primers and their sequences used in diagnosis of *U. virens*

Primer name	Primer sequence	Position (bp)
ITS4 (Reverse)	5’-TCCTCCGCTTATTGATATGC-3’	2390-2409
ITS5 (Forward)	5’-GGAAGTAAAAGTCGTAACAAGG-3’	1737-1758

Table 3.5: Composition of reaction mixture (30 µl) used in PCR for detection of *U. virens*

Components	Stock Solution	Volume	Final Concentration
Template DNA	25 ng/µl	2.0 µl	50 ng/µl
Primer (R)	10 µM	1.5 µl	1 µM
Primer (F)	10 µM	1.5 µl	1 µM
GoTaq™ DNA polymerase (Promega Inc.)	5 units/ µl	0.4 µl	2 units/ µl
Go Taq ™ buffer (Green) (Promega Inc.)	5X	6.0 µl	1X
$MgCl_2$ (Promega Inc.)	25 mM	2.4 µl	2.0 mM
dNTP's (Promega Inc.)	10 mM	0.6 µl	0.2 mM
Nuclease free Water (Promega Inc.)	-	12.6 µl	-
Dimethyl sulfoxide (DMSO)	-	3 µl	
Total	-	30 µl	-

Table 3.6: PCR amplification protocol with universal ITS primers used for identification of *U. virens*

Sr. No.	Temperature	Duration	
1	96°C (Initially denaturation)	2min	
2	96°C (Denaturation)	20 secs	30 cycles
3	53°C (Annealing)	30 secs	
4	72°C (Extension)	30 secs	
5	72°C (Final extension)	7 min	

Table 3.7: Nested PCR primers and their sequences used in diagnosis of *U. virens*

Primer name	Primer sequence
US2-5 (Forward)	5′-CAATGCATGTCTGAGTGGATTTTTG-3′
US4-3 (Reverse)	5′-CCAACACCAAGCGCAAGACAGA-3′

Table 3.8: PCR amplification protocol with nested PCR primers used for identification of *U. virens*

Sr. No.	Temperature	Duration
1	96°C (Initially denaturation)	2min
2	96°C (Denaturation)	20 secs
3	58°C (Annealing)	30 secs
4	72°C (Extension)	30 secs
5	72°C (Final extension)	7 min

30 cycles

3.6 Visualization of PCR product

After amplification, 8µl of each sample was loaded in 0.8% agarose gel prepared on 0.5X TBE buffer. For agarose gel preparation, 2.4 g of agarose was dissolved in 300 ml of 0.5X TBE buffer (Tris base – 45 mM, Boric acid – 45 mM and EDTA – 1mM). The mixture was heated till agarose dissolved completely i.e. when the solution became transparent and clear. It was cooled down to 60°C with constant stirring. Ethidium bromide was added to a final concentration of 0.5 µl/ml of buffer. Then the agarose solution was poured into an already prepared gel mould with combs and was left for 20-30 mins for solidification. When the gel solidified, the DNA samples were loaded into wells with the help of multi-channel pipette (Thermo Scientifc). Along with DNA samples, 5µl of KB base pair ladder or 100 base pair DNA ladder (Promega Inc.) was also loaded. PCR products were resolved by gel electrophoresis at 5V/cm for 3h. The gels were visualized under UV light and photographed using SYNGENE gel documentation system with "GeneSnap" software programme. PCR-amplified products were analyzed by standard agarose gel electrophoresis (Sambrook *et al* 1989).

3.7 Screening of rice germplasm accessions under field conditions for identification of potential donors imparting resistance to false smut

3.7.1 Raising of rice germplasm accessions under field conditions

A set of 212 diverse lines of rice germplasm were sown in the experimental area, Department of Plant Breeding and Genetics on 2nd June 2017. The accessions belong to various subpopulation groups within *Oryza sativa* representing the genetic diversity among the panel (Table 3.9). Based on the days to 50 per cent of flowering, the lines were categorized into four groups (Table 3.10). These lines belong to the 2K panel from International Rice Research Institute, Philippines which represents the diverse groups of

cultivated rice from various geographical and ecological regions of rice growing countries all over the world (Table 3.11). The one-month-old nursery was transplanted at the Experimental area, Department of Plant Breeding and Genetics, PAU, Ludhiana, Punjab. Each germplasm line was transplanted into a paired row, having ten plants in each row with the spacing of 15×20 cm. The susceptible checks GSR 123 (breeding line) and PR 116 (commercial variety) were planted after every 20 germplasm lines. The fertilizer application and other standard cultural operations were carried out as per the recommendation of the PAU, Ludhiana for rice (Anonymous 2017). The germplasm lines were screened under field conditions by spraying the spore suspension of *U. virens* at boot stage of the crop. The spore suspension was prepared as mentioned in section 3.2. The various observations on disease variables were recorded (section 3.7.2).

Table 3.9: Number of genotypes belonging to various sub-populations of *Oryza sativa*

Subpopulations	Number of genotypes
Indica	22
Tropical japonica	74
Temperate japonica	12
Aromatic	1
Aus	8
Admixed-japonica	27
Admixed	12
NA	60

Table 3.10: Grouping of genotypes based on days to 50 per cent flowering

Group	Number of genotypes	Duration (days)
Very short	40	70-90
Short	111	91-105
Medium	39	106-115
Long	26	>115
Total	216	

Table 3.11: List of germplasm lines (2K Panel, IRRI) screened under field conditions for resistance to false smut

S. No.	Designation	D 50% f
1	CHIGYUNGDO::IRGC 55466-1	68
2	YAKUMO::IRGC 5320-1	72
3	ZHENSHAN 97 B	74
4	YAN ZHAO 9::IRGC 63062-1	74
5	CO 39::IRGC 51231-1	76
6	YANGKUM (RED)::IRGC 32406-1	91
7	AEDAL::IRGC 55441-1	74
8	GHASELU MAP::IRGC 72527-1	79
9	NIPPONBARE	79
10	DUMAI::IRGC 25852-1	79
11	KAM MRA::IRGC 62172-1	79
12	IR 73678-20-1-B::IRGC 117292-1	76
13	SADU CHO	76
14	M 202	74
15	BR IRGA 409::IRGC 116960-1	114
16	KUROKA::IRGC 74556-C1	85
17	IRAT 144::IRGC 55685-C1	82
18	WARABEHATOMOCHI::IRGC 14779-1	82
19	GOGO LEMPAK::IRGC 43392-C1	81
20	RIZZOTTO 264::IRGC 65727-1	82
21	WAB 368-B-1-H1-HB::IRGC 117359-1	82
22	DAWASAM (RED)::IRGC 32389-1	84
23	RIENALDO BERZANO::IRGC 3230-1	84
24	LI JIANG XIN TUAN HEI GU	87
25	DULAR	90
26	SHADA SHAITA::IRGC 64796-1	82
27	EDOGAWA::IRGC 74468-1	82
28	INDIO::IRGC 116998-1	83
29	CHAHORA 144::IRGC 27869-1	85
30	DA 28::IRGC 6246-1 (4246-1 ?)	87
31	INDANE::IRGC 33130-1	114
32	ARGO::IRGC 82418-1	83
33	BREVIARISTATA (RAIA)::IRGC 3189-1	86

S. No.	Designation	D 50% f
34	KHUDWANI ACC 409::IRGC 34216-1	86
35	KATAKTARA DA 2::IRGC 32565-1	88
36	N 22	101
37	RP 1451-1196-1562-4218::IRGC 117351-1	88
38	NSICRC 106::IRGC 117370-1 (17370-1 ?)	88
39	PICONEGRO::IRGC 117022-1	82
40	GHARIB::IRGC 32306-1	83
41	ITA 235::IRGC 64854-1	92
42	DA 8::IRGC 6422-1	92
43	BRAZOS::IRGC 24273-1	92
44	CR 5272::IRGC 116971-1	99
45	EMBRAPA 6 CHUI::IRGC 116981-1	82
46	LATSIKA::IRGC 69367-1	82
47	JIMBRUK JOLOWORO::IRGC 43420-C1	88
48	BARAN BORO::IRGC 27509-1	95
49	KEN CHIAO JU HSIAO LI::IRGC 1217-1	95
50	1-52-6::IRGC 39111-1	95
51	BOSSA::IRGC 57781-1	95
52	LUDAN::IRGC 64189-C1	95
53	CIRAD 409::IRGC 116969-1	95
54	VANDANA::IRGC 117398-1	95
55	MINGHUI 63	95
56	CIMARRON::IRGC 116967-1	99
57	PALMIRA::IRGC 5097-1	104
58	IAC 164::IRGC 117251-1	104
59	DOONGARA::IRGC 78392-1	99
60	KETAN KONIR::IRGC 43444-C1	99
61	TRES MESES::IRGC 6464-C1	104
62	DA YE XI NAN::IRGC 73958-1	100
63	KURULU WEE (WHITE)::IRGC 66518-1	101
64	UCHUTI::IRGC 14694-1	101
65	RHS 107-2-1-2TB-1JM::IRGC 117025-1	101
66	MARS::IRGC 38541-1	101
67	SANHUANGZHAN NO 2	91
68	AZUCENA	101

S. No.	Designation	D 50% f
69	GUATEMALA 1021::IRGC 3388-1	101
70	IAC 120::IRGC 22712-1	101
71	GUAYQUIRARO P A::IRGC 116987-1	101
72	TOS 10483::IRGC 56723-1	101
73	TOX 1011-4-1::IRGC 117033-1	101
74	TX 10438::IRGC 117035-1	101
75	BAKILIKINDA::IRGC 63121-1	101
76	PACHOLINHA::IRGC 50531-1	101
77	ORIENTE 10::IRGC 55808-1	101
78	MATAHAMBRE::IRGC 53200-1	107
79	CAIAPO::IRGC 116962-1	101
80	IC 27525::IRGC 53989-1	101
81	ARC 10317::IRGC 12430-1	102
82	GHARIB::IRGC 32303-1	102
83	AUS JOTA::IRGC 66767-1	102
84	BAKUNG (H)::IRGC 60220-1	102
85	JAMAICA 3::IRGC 4105-1	102
86	ARC 12701::IRGC 22267-1	102
87	MUT IAC 25-44-807::IRGC 68799-1	102
88	JI BO YA::IRGC 77446-1	102
89	CYPRESS	99
90	BICO BRANCO::IRGC 38994-1	99
91	CANA ROXA::IRGC 25966-1	102
92	KALAMKATI::IRGC 45975-1	102
93	MIKHUDEB::IRGC 25892-1	102
94	ARC 12451::IRGC 41052-1	99
95	KIKILONG::IRGC 71539-1	99
96	TOS 724::IRGC 11108-1	104
97	HAWM OM::IRGC 23729-1	104
98	IAC 1111::IRGC 39050-1	104
99	KHAO KAP SANG::IRGC 23423-1	104
100	EL PASO L 227::IRGC 116979-1	104
101	NATO::IRGC 1819-1	104
102	KHAO DAM::IRGC 23385-1	104
103	KINANDANG PATONG::IRGC 23364-1	104

S. No.	Designation	D 50% f
104	MA HAE::IRGC 23754-1	104
105	RT 1031-69::IRGC 15092-1	104
106	TCHAMPA::IRGC 32362-1	100
107	HEI CHIAO CHUI LI HSIANG KENG::IRGC 1112-1	88
108	SAYLLEBON::IRGC 32509-1	100
109	MAINTIMOLOTSY 1226::IRGC 11010-C1	100
110	BAMAWPYAN::IRGC 72458-1	100
111	TAINUNG 67	100
112	DINOLORES::IRGC 67431-1	100
113	IR 64-21	100
114	DOMSIAH::IRGC 32292-1	100
115	BG 301::IRGC 117315-1	100
116	62-667::IRGC 15147-1	100
117	RXAR RGUE::IRGC 1943-1	100
118	63-104::IRGC 15100-C1	100
119	IA CUBA 23::IRGC 116991-1	100
120	DOM SUFID	100
121	VARY LAVA::IRGC 386-1	100
122	TORO 2::IRGC 66761-1	100
123	MALAGKIT PUTI::IRGC 19451-1	100
124	IR 65482-17-511-5-7::IRGC 117284-1	100
125	GOGO::IRGC 43390-C1	100
126	GSR105 (S)	100
127	GSR123 (S)	100
128	SAYARI::IRGC 74716-1	100
129	ARC 11495::IRGC 21431-1	100
130	IR 74371-3-1-1::IRGC 117373-1	100
131	IAC 47::IRGC 116992-1	100
132	TOANG::IRGC 19144-1	102
133	TAINUNG 29::IRGC 65309-1	102
134	TOS 5790::IRGC 117256-1	102
135	IR 77298-14-1-2::IRGC 117374-1	102
136	JHONA 26::IRGC 27967-1	102
137	CCT 3-37-3-3-3-1::IRGC 117322-1	108
138	KHAO DO NGOI::IRGC 29772-1	108

S. No.	Designation	D 50% f
139	PULU LAPA::IRGC 48857-C1	104
140	LAMBAYQUE 1::IRGC 10769-1	108
141	TCHAMPA::IRGC 32368-1	103
142	BAGANAN ASALAO::IRGC 71503-1	108
143	SILADON::IRGC 71620-1	103
144	PERLA::IRGC 117021-1	107
145	CAPI 93::IRGC 116964-1	107
146	GANIGI::IRGC 48698-C1	104
147	PADI KOMPAI::IRGC 25510-1	97
148	OS 4::IRGC 11335-C1	97
149	MIMIDAM::IRGC 25897-1	110
150	CT 6946-9-1-2-M-1P::IRGC 117329-1	92
151	C 8434::IRGC 13496-1	104
152	IR 72132-AC 6-1::IRGC 117369-1	106
153	ICTA POLOCHIC::IRGC 116997-1	105
154	INIAP 415::IRGC 117001-1	107
155	IR 60080-46 A::IRGC 117396-1	103
156	BENGIZA::IRGC 69845-1	103
157	VILLAGUAY P A::IRGC 117259-1	103
158	NS 1288::IRGC 68930-1	103
159	B 6144 F-MR-6::IRGC 117313-1	103
160	PITIPO::IRGC 117023-1	111
161	MAHAPLEU (504)::IRGC 50865-1	108
162	CT 9993-5-10-1-M::IRGC 116974-1	108
163	D 4-136::IRGC 31051-1	108
164	RANAU KADAI::IRGC 71604-1	108
165	J 104::IRGC 117008-1	108
166	ARC 11294::IRGC 21296-1	108
167	GOMPA 2::IRGC 12894-1	108
168	DAKPA::IRGC 64888-1	108
169	FOSSA HV::IRGC 16069-1	108
170	VASSE NANAN::IRGC 56812-1	111
171	MOROBEREKAN	111
172	NILO 3 B::IRGC 10283-1	109
173	YANCAOUSSA::IRGC 16071-C1	105

S. No.	Designation	D 50% f
174	GBANTE::IRGC 16081-1	108
175	GAO GAN DA NUO::IRGC 73974-1	107
176	KAKANI 2::IRGC 13373-C1	113
177	JUMA 51::IRGC 117009-1	116
178	TAK SIAH::IRGC 73126-1	109
179	PAE URA::IRGC 27321-1	116
180	SPR 87032-2-1-1-4::IRGC 117355-1	110
181	ONDEYKAM::IRGC 67846-1	110
182	IR 43::IRGC 117005-1	110
183	IR 77384-12-35-3-12-1-B::IRGC 117299-1	116
184	NHTA 5::IRGC 186-1	106
185	ICTA PAZOS::IRGC 116996-1	114
186	GEMJYA JYANAM::IRGC 32411-C1	106
187	DOM ZARD::IRGC 12881-1 (1288-1 ?)	116
188	RATHAL::IRGC 31525-1	116
189	EPAGRI 109::IRGC 116983-1	116
190	CINA::IRGC 27116-1	113
191	BATUKURU WEE::IRGC 67614-1	119
192	DAVAO::IRGC 8244-C1	119
193	REXORO::IRGC 1715-1	102
194	ABRI::IRGC 32380-1	127
195	WALANGA::IRGC 27502-1	117
196	PANAMA 1537::IRGC 117019-1	125
197	IR 80314-4-B-1-3-B::IRGC 117308-1	130
198	NENG NAH::IRGC 78275-1	116
199	OIRAN::IRGC 8257-1	116
200	RATHAL::IRGC 31524-1	119
201	NAVOLATO A 71::IRGC 117016-1	127
202	KHAU MEO::IRGC 78330-1	119
203	NEP ME HOA BINH::IRGC 78366-1	127
204	JUMALI::IRGC 9542-C1	130
205	SAMBA MAHSURI::IRGC 117377-1	125
206	KHAO' MUM::IRGC 78259-1	125
207	CR 8334::IRGC 116972-1	130
208	PTB 25::IRGC 6386-1	120

S. No.	Designation	D 50% f
209	RYT3229 (PAU3835-12-1-1-2) (Sus)	112
210	PR116 (S)	112
211	GSR105 (S)	100
212	GSR123 (S)	100

3.7.2 Data assessment

The plants in the field were monitored for disease development and following disease variables and other parameters were recorded.

1. Number of tillers per plant
2. Per cent infected panicles
3. Number of smut balls per panicle
4. Number of smut balls per plant
5. Visual disease score (Standard Evaluation System for Rice, 2013) (Table 3.13)
6. Days to 50 per cent flowering

The per cent infected panicle was recorded as per the following formula:

$$\textbf{Per centinfected panicle} = \frac{\text{Number of infected panicles}}{\text{Total number of panicles}} \times 100$$

Disease score on 0-9 scale were calculated following standard evaluation system for rice (IRRI, 2002) (Table 3.12)

Table 3.12: Rating scale of false smut of rice on the basis of infected panicles (IRRI, 2002)

Visual score	Infected panicle
0	0
1	<1%
3	1.1-5%
5	5.1-25%
7	25.1-50%
9	>50%

Based on the visual score the lines were categorized as resistant (R), moderately resistant (MR), moderately susceptible (MS) and susceptible (S) (Table 3.13).

Table 3.13: Grouping based on visual disease score

Category	Visual disease score
Resistant	0
Moderately resistant	1-3
Moderately susceptible	3.1-5
Susceptible	5.1-9

3.7.3 Screening of selected resistant accessions under controlled conditions

The germplasm lines showing resistance to false smut under field conditions (Table 3.14) were sown at the experimental area, Department of Plant Breeding and Genetics, PAU, Ludhiana. These resistant lines were along with two susceptible checks GSR 123 (breeding line) and PR 116 (commercial variety) were selected for this experiment (Table 3.14). Most of the selected resistant lines are short duration followed by medium duration and only two long duration accessions. The one month old nursery of rice lines was transplanted in the fields, with three replications. Later, before boot stage of crop, they were transferred to pots in growth chamber room 3, School of Agricultural Biotechnology, PAU, Ludhiana (Plate 7). The fertilizer application and other operations were carried out as per the recommendations of PAU, Ludhiana for rice (Anonymous 2018). The pots were maintained at 26°C for 5 days and transferred to greenhouse condition in a completely randomized block design (CRD). The relative humidity (> 95%) was maintained artificially by the humidifier equipped in the chamber room (Plate 7). The preparation of inoculum and inoculation method was carried out as mentioned above in section 3.2. Disease assessment and analysis was done as mentioned in section 3.7.2.

3.8 Quantitative trait loci mapping for resistance to false smut in rice

The SSR markers from the universal core genetic map of rice were used for detecting polymorphism between the parents. Seventy-eight SSR primer pairs, which were distributed on all of the 12 rice chromosomes, were used on parents. The polymorphic markers so selected were chosen to genotype plants in the F_2 population. SSRs were scored visually on the basis of their presence or absence and size in each of the two parents and F_2 plants. The marker data generated were used for QTL analysis. The flow-chart for plan of work is listed in Figure 3.1 and Figure 3.2.

3.8.1 Preparation of crosses between diverse parents

From the 212 rice accessions, the resistant lines after screening under field as well as artificial conditions were chosen for selection of resistant donor in preparation of crosses. The diverse parents selected for the cross and the plan of six prepared crosses are listed in Table 3.15.

Plate 7: Screening of selected resistant accessions under controlled conditions (a) Growth chamber room (b) Incubation of plants after inoculation.

Table 3.14: List of selected germplasm lines (2K Panel, IRRI) for screening under controlled conditions

2020	Designation	IRGC No.
1	BARAN BORO::IRGC 27509-1	117434
2	KEN CHIAO JU HSIAO LI::IRGC 1217-1	117512
3	CR 5272::IRGC 116971-1	120918
4	BG 301::IRGC 117315-1	120892
5	VARY LAVA::IRGC 386-1	121685
6	TORO 2::IRGC 66761-1	124468
7	IAC 47::IRGC 116992-1	121361
8	DA YE XI NAN::IRGC 73958-1	124475
9	DOMSIAH::IRGC 32292-1	117467
10	TAINUNG 29::IRGC 65309-1	121644
11	MUT IAC 25-44-807::IRGC 68799-1	121672
12	CANA ROXA::IRGC 25966-1	117440
13	IR 60080-46 A::IRGC 117396-1	117596
14	HAWM OM::IRGC 23729-1	117484
15	GAO GAN DA NUO::IRGC 73974-1	121340
16	DAKPA::IRGC 64888-1	121563
17	TCHAMPA::IRGC 32362-1	117585
18	HEI CHIAO CHUI LI HSIANG KENG::IRGC 1112-1	121690
19	ITA 235::IRGC 64854-1	121368
20	PADI KOMPAI::IRGC 25510-1 TJ	124490
21	REXORO::IRGC 1715-1 TJ	117568
22	GEMJYA JYANAM::IRGC 32411-C1	121739
23	ARC 11294::IRGC 21296-1	121658
24	KHAO DO NGOI::IRGC 29772-1	121208
25	TAK SIAH::IRGC 73126-1	121224
26	KAKANI 2::IRGC 13373-C1	122128
27	INDANE::IRGC 33130-1	117492
28	GSR123 (S)	
29	PR116 (S)	

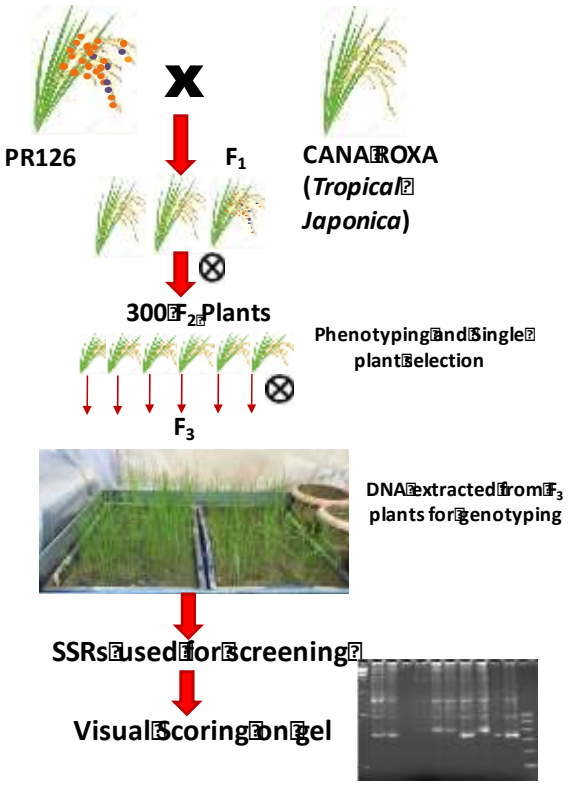


Fig. 3.1: Flow-chart for QTL analysis in mapping population derived from the cross between the parents PR126 and CANA ROXA

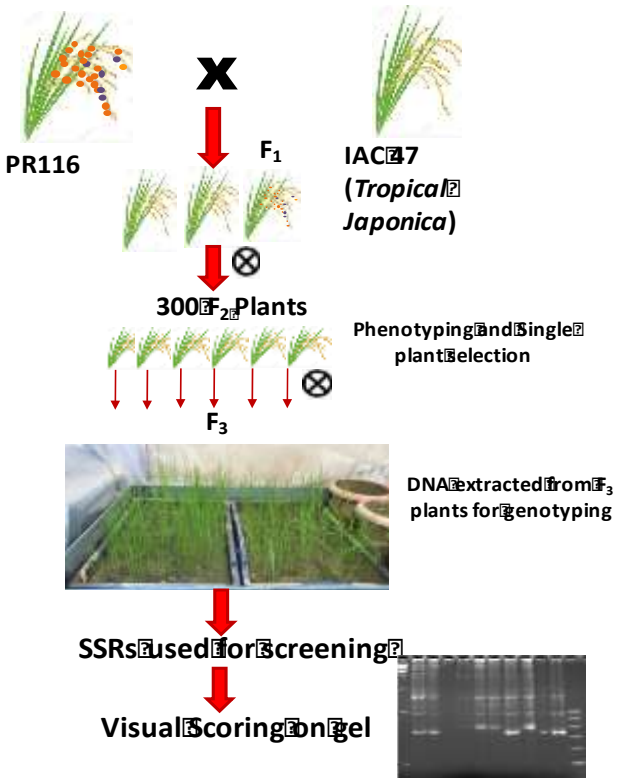


Fig. 3.2: Flow-chart for QTL analysis in mapping population derived from the cross between the parents PR116 and IAC 47

Table 3.15: Selection of parents for preparation of crosses

Female	Male
GSR123	CR5272 (*indica*)
PR116	IAC47 (*Tropical japonica*)
PR116	CANA ROXA
PR126	CR5272 (*indica*)
PR126	IAC47 (*Tropical japonica*)
PR126	CANA ROXA

3.8.2 Mapping population

The certain cross with good agronomic traits of their progeny were maintained and forwarded to F_1 generation to raise mapping population. The crosses selected were PR126 × CANAROXA and PR116 × IAC 47. The recipient plants used in the crosses (PR126 and PR116) were emasculated at anthesis stage and covered with polythene bags to prevent their pollination. The pollen of the CANAROXA and IAC 47, which were used as donor in the above crosses, was collected in the noon and used to pollinate PR126 and PR116, respectively. The pollinated plants were covered with polythene bags allowing the pollen to fertilize the ovaries of plants. The seeds of the F_1 were collected which was futher used to raise its nursery. The one month old nursery of F_1 was transplanted in the fields (Plate 8). The sowing of mapping population and other cultural operations were carried out as per the recommendations of PAU, Ludhiana for rice (Anonymous 2018). The F_2 seeds were selected separately from each plant in properly labelled bags.

3.8.3 Phenotyping of F_1 and F_2 mapping population

The data about the reaction of the plant was taken in accordance with the disease rating scale (Table 3.12). Disease score on 0-9 scale were calculated following standard evaluation system for rice (IRRI, 2002). The plant disease reaction was also grouped as Resistant, Moderately resistant, Moderately susceptible and Susceptible based on the disease visual score (Table 3.13).

3.8.4 Genotyping of F_3 mapping population

The F_3 population was grown and maintained under controlled conditions in trays. Each plant was marked with a properly labelled tag. The leaves of the individual plant were sampled at 5 leaf stage for DNA extraction. The DNA extraction was carried out by using Cetyl trimethyl ammonium bromide (CTAB) method (section 3.3.4, Sambrook *et al* 1989). The SSR markers from the universal core genetic map of rice were used for detecting

polymorphism between the parents. Seventy-eight SSR primer pairs, which were distributed on all of the 12 rice chromosomes, were used on parents. SSRs were scored visually on the basis of their presence or absence and size in each of the two parents and F_2 plants. The allelic data of polymorphic SSR markers were used to access the genetic diversity in the study material.

(a)

(b)

(c)

(d)

Plate 8: Field view of F_2 mapping population (a,b) Nursery, (c,d) After transplanting.

CHAPTER IV

RESULTS AND DISCUSSION

False smut of rice causes quantitative as well as qualitative losses in yield of rice. The current study was carried out in 2018-2020 to understand the role of primary inoculum which causes the preliminary infection of rice and focuses on selection of resistant donors by screening of rice germplasm accessions and mapping QTL for resistance to false smut in rice.

4.1 Determining the role of seed as primary source of inoculum causing false smut disease in rice

The susceptible check PR116 was used in this study. Two treatments were maintained one with healthy seed (T1) and the other with infected seed (T2). Out of twenty plants from T1 (healthy seeds), none of the plants were found to be infected and show visual symptoms of false smut of rice (Table 4.1). On the other hand, only one plant out of twenty from T2 (infected seeds) showed symptoms of false smut. T1 (healthy seeds) served as a control treatment. Visual scoring of the infected plant was done and the diseased plant showed the score of 5 (Table 4.1).

Per cent infected panicles was found to be 0.1% (Table 4.1) in infected plant from infected seed. The number of smut balls per panicle was 1 in infected plant from T2 (infected seeds). Per cent infection in plants from infected seed was 5% as compared to 0% in plants from healthy seeds (Fig 4.1). The results imply that the seed rarely serve as a source of primary infection caused by *U. virens* in causing false smut of rice in otherwise susceptible cultivar PR116.

4.2 Determining the role of soil as primary source of inoculum causing false smut disease in rice

The healthy seed of PR116 were selected and grown in earthen pots containing infected soil (T3). The earthen pots containing sterilized soil was used as check (T4). Out of twenty plants from T3 (sick soil), nine plants were found to be infected and show visual symptoms of false smut of rice. On the other hand, only two plants out of 20 from T4 (sterilized soil) showed symptoms of false smut (Table 4.2). Visual scoring of the infected plant was done and seven plants in T3 (sick soil) recorded disease score of 5. (Table 4.2). These seven plants were grouped as moderately susceptible based on the disease score of 5 while the other two plants were grouped as susceptible with the disease score of 7. The infected plants in T4 (sterilized soil) were grouped as moderately susceptible based on the disease score of 5. Per cent infected panicles was found to be in range of 9-33% in T3 (sick soil, Table 4.2). On the other hand, per cent infected panicles in T4 (sterilized soil) were 8-

13%. The number of smut balls per panicle was in the range of 1.0-2.5 in T3 (sick soil) as compared to 0.0-1.0 in T4 (sterilized soil). Per cent infection in plants from infected soil was 45% as compared to 10% in sterilized soil (Fig 4.1). The results showed that soil serve as a reservoir of *U. virens* inoculum for initiating the primary infection under the suitable environmental conditions. The chlamydospores are thick-walled resting structures which can survive in the soil up to various months during offseason and cause infection after germination when the rice host and suitable environmental conditions set in.

Table 4.1: Effect of infected and healthy seed on the infection of false smut of rice

Plant No.	Healthy seed (T1)				Treated/infected seed (T2)			
	TP	%IP	NSB/Panicle	Score	TP	%IP	NSB/Panicle	Score
1	11	-	-	-	10	**10**	**1.0**	**5**
2	10	-	-	-	11	-	-	-
3	9	-	-	-	13	-	-	-
4	12	-	-	-	8	-	-	-
5	11	-	-	-	10	-	-	-
6	11	-	-	-	12	-	-	-
7	8	-	-	-	10	-	-	-
8	12	-	-	-	10	-	-	-
9	5	-	-	-	12	-	-	-
10	10	-	-	-	9	-	-	-
11	6	-	-	-	11	-	-	-
12	9	-	-	-	14	-	-	-
13	11	-	-	-	10	-	-	-
14	14	-	-	-	12	-	-	-
15	9	-	-	-	12	-	-	-
16	12	-	-	-	6	-	-	-
17	11	-	-	-	11	-	-	-
18	11	-	-	-	13	-	-	-
19	10	-	-	-	8	-	-	-
20	9	-	-	-	11	-	-	-

TP: Total panicles, % IP: Per cent infected panicle; NSB/P: Number of smut balls/panicles
Score: Disease score

Table 4.2: Effect of sick and sterilized soil on the infection of false smut of rice

Plant No.	Sick soil (T3)				Sterilized soil (T4)			
	TP	%IP	NSB/ Panicle	Score	TP	% IP	NSB/ Panicle	Score
1	6	33	1.0	**7**	12	8	1.0	**5**
2	10	10	1.0	**5**	8	13	1.0	**5**
3	8	25	1.0	**5**	12	-	-	-
4	12	25	1.3	**5**	11	-	-	-
5	11	18	1.0	**5**	11	-	-	-
6	9	11	1.0	**5**	6	-	-	-
7	10	30	2.5	**7**	12	-	-	-
8	11	9	1.0	**5**	10	-	-	-
9	11	9	1.0	**5**	15	-	-	-
10	5	-	-	-	12	-	-	-
11	7	-	-	-	8	-	-	-
12	12	-	-	-	12	-	-	-
13	9	-	-	-	11	-	-	-
14	11	-	-	-	11	-	-	-
15	9	-	-	-	6	-	-	-
16	12	-	-	-	12	-	-	-
17	10	-	-	-	10	-	-	-
18	5	-	-	-	15	-	-	-
19	7	-	-	-	9	-	-	-
20	12	-	-	-	10	-	-	-

TP: Total panicles, % IP: Per cent infected panicle; NSB/P: Number of smut balls/panicles
Score: Disease score

Realizing the importance of *U. virens* inoculum in soil, Ashizawa *et al* (2010) and Li *et al* (2013) developed a rapid and sensitive technique for detection and quantification of *U. virens* with the species-specific primers and real time PCR respectively, from the soils of paddy fields. This was then compared with the visual assessment of symptoms in the field crop.

4.3 Determining the role of air-borne spores as primary source of inoculum causing false smut disease in rice

The susceptible check PR116 was used in this study. The plants were not covered with bags before the panicle emergence stage to allow the air borne inoculum infecting the plant (T5). The plants covered with bags were used as control (T6). Out of twenty plants from T5 (uncovered plants), eleven plants were found to be infected and show visual symptoms of false smut of rice (Table 4.3). On the other hand, only one plant out of 20 plants from T6 (covered plants) showed symptoms of false smut. Visual scoring of the infected plants was done and six plants showed disease score of 5 while five plants showed disease score of 7 in T5 (uncovered plants, Table 4.3). The six plants with disease score of 5 were grouped as moderately susceptible while the other five plants were grouped as susceptible based on the disease score of 7. Per cent infected panicles was found to be in range of 10-36% in case of T5 (uncovered plants) as compared to 9% in case of T6 (covered plants, Table 4.3). The number of smut balls per panicle was in the range 1.0-2.5 in T5 (uncovered plants) as compared to 0.0-1.0 in case of T6 (covered plants).

Per cent infection in uncovered plants was 55% as compared to 5% (Fig 4.1) in case of covered plants. These results imply that the air-borne inoculum serve as an important source for primary infection caused by *U. virens* in causing false smut of rice in susceptible cultivar PR116. The 55% infection in uncovered plants (Fig 4.1) reveals that the air-borne inoculum in the form of conidia as well as ascospores might be responsible for causing infection of crop at boot stage, the most susceptible stage for pathogen to cause infection.

The per cent infection was more pronounced in case of air-borne experiment (Figure 4.1) implying the role of air-borne conidia in development and spread of false smut disease especially at the booting stage. Moreover, the high per centinfection in case of soil borne experiment (Figure 4.1) implies the role of soil-borne chlamydospores/overwintered sclerotia in causing primary infections. The results showed that pathogen does not survive in/on the seed and seed do not serve as primary source of infection.

Kumari (2014) found that the sclerotia and chlamydospores were found to be viable only up to three months of storage and gradually loses its viability with time. The seeds inoculated with chlamydospore also could not produce disease symptoms. The study supports the above results that seed do not serve as primary source of inoculum in causing false smut of rice.

The role of sclerotia in soil under natural field conditions in causing primary infection is very rare as the high temperature during hot and dry months of summer under Indian conditions render the propagules non-viable for germination and subsequently, infection of

the host plant. The chlamydospores, produced on the surface of smut balls of rice serve as the important source of inoculum between the seasons of rice, germinates and produce conidia to cause secondary infection of rice (Zhang *et al* 2003). The chlamydospores in the soil are believed to germinate and cause infection of the rice seedlings (Ikegami 1962).

Table 4.3: Effect of air-borne inoculum on the infection of false smut of rice

Plant No.	Uncovered panicle (T5)				Covered panicle (T6)			
	TP	% IP	NSB/Panicle	Score	TP	% IP	NSB/Panicle	Score
1	12	25	1.0	5	11	**9**	1.0	**5**
2	9	22	1.0	5	9	-	-	-
3	9	33	1.0	7	11	-	-	-
4	12	33	1.3	7	11	-	-	-
5	10	10	1.0	5	10	-	-	-
6	11	27	1.0	7	8	-	-	-
7	11	36	2.5	7	11	-	-	-
8	10	10	1.0	5	12	-	-	-
9	9	22	1.0	5	11	-	-	-
10	15	20	1.0	5	11	-	-	-
11	9	33	1.0	7	10	-	-	-
12	9	-	-	-	11	-	-	-
13	15	-	-	-	11	-	-	-
14	9	-	-	-	10	-	-	-
15	11	-	-	-	12	-	-	-
16	7	-	-	-	9	-	-	-
17	8	-	-	-	12	-	-	-
18	11	-	-	-	12	-	-	-
19	16	-	-	-	13	-	-	-
20	13	-	-	-	8	-	-	-

TP: Total panicles, % IP: Per cent infected panicle; NSB/P: Number of smut balls/panicles
Score: Disease score

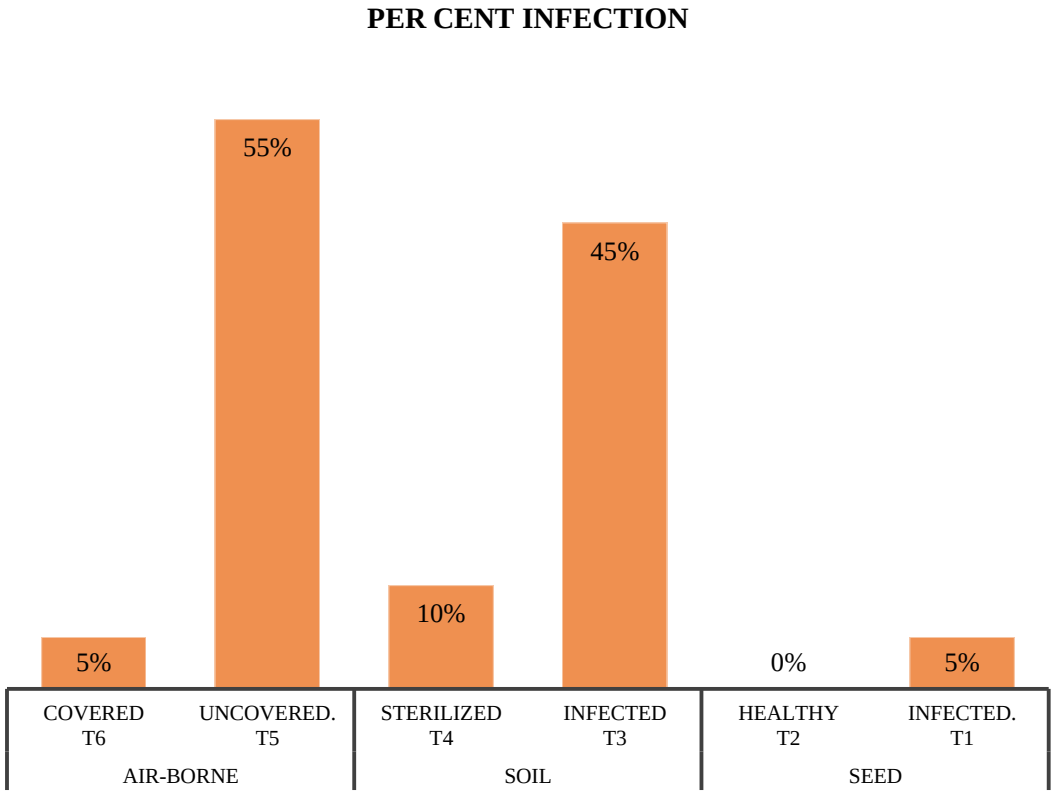


Fig. 4.1: Data representing the role of different primary inoculum treatments under this study in development of false smut in rice

TeBeest *et al* (2010) also found that the fungus is soil-borne in the form of spore balls found in the field heavily infested with false smut. These findings are in accordance with the above results of the current research that the fungus is soil-borne in nature and later on it invades the seedlings of rice.

Ashizawa *et al* (2010) provides an efficient evidence about the role of soil-borne chlamydospores in causing primary infection of the rice plants. TeBeest *et al* (2010) reported that increasing the *U. virens* inoculum levels in the soil increases the infection levels of rice.

Tanaka *et al* (2017) also found that the fungus is found in the soil in the form of thick-walled conidia and nested PCR species-specific primers reveals the presence of *U. virens* in rice roots and apices at vegetative stage of plant.

The present study will be helpful in mitigating the risk management of false smut disease by development of suitable management strategies such as crop rotation and soil treatment with fungicides before sowing to check the soil-borne inoculum of *U. virens.* Realizing the importance of air-borne inoculum at the boot stage of crop, the use of fungicides especially copper-based fungicides is highly recommended before heading for the management of false smut under field conditions.

4.4 Screening of 212 rice germplasm accessions under field conditions for identification of potential donors imparting resistance to false smut

The genetic material for this study comprised of a set of diverse germplasm collection (212 germplasm accessions) collected from International Rice Research Institute (IRRI), Philippines. The accessions belong to the 2K panel, which represents the range of genetic diversity found in cultivated rice, from around the world. It covers almost all the *Oryza sativa* groups collected from various geographical and ecological regions across the rice-growing countries of the world. The lines belongs to various sub populations- *indica, tropical japonica, temperate japonica, aromatic, aus, admixed-japonica* and *admixed.* The data pertaining to the screening of 212 rice germplasm accessions for identification of potential donors imparting resistance to false smut under field conditions in 2017 at Ludhiana is given in Table 4.4. Quantitative variation in disease resistance was found in all the lines. The various variables such as days to 50% flowering, total panicles per plant, per centinfected panicles and number of smut balls per panicle was recorded.

The total panicles per plant were mostly recorded in the range 16-22 (Figure 4.2). The minimum value of 7 panicles per plant was observed in five lines (Figure 4.2).

The number of smut balls per plant is a measure of disease severity and is related to the level of resistance in the cultivar of rice. It ranges from 0-12.6 with mean value of 0.58. Out of 212 lines, about 36 lines showed higher number of smut balls per plant above mean value. The highest number of smut balls were observed on susceptible checks GSR105 (12.6) followed by GSR 123 (11.2, Table 4.4). The maximum lines (198) were observed without any smut ball and disease (Figure 4.3) and around 67 lines show smut ball in the range 1-2 smut balls per panicle (Figure 4.3).

The per cent infected panicle is a measure of quantitative disease measurement. It ranged from 0-31.3% (Table 4.4.) with mean value of 2.1%. Out of 212 lines screened, maximum lines were observed with zero per cent infected panicle (Figure 4.4). The highest percentage of infected panicle was observed in RP1451 (31.3%, Figure 4.4) followed by CAPI93 (20.2%) and susceptible check GSR123 (17.9%).

Out of 212 rice accessions, 81 were found to show disease symptoms while 131 accessions were found to be completely free from the disease with disease score of 0 (Figure 4.5). According to the disease score, the plants were divided into different disease reaction groups (Figure 4.6). About 131 rice accessions with disease score of 0 were grouped as resistant while 73, 7 and 1 line was grouped as moderately resistant, moderately susceptible and susceptible, respectively (Figure 4.6).

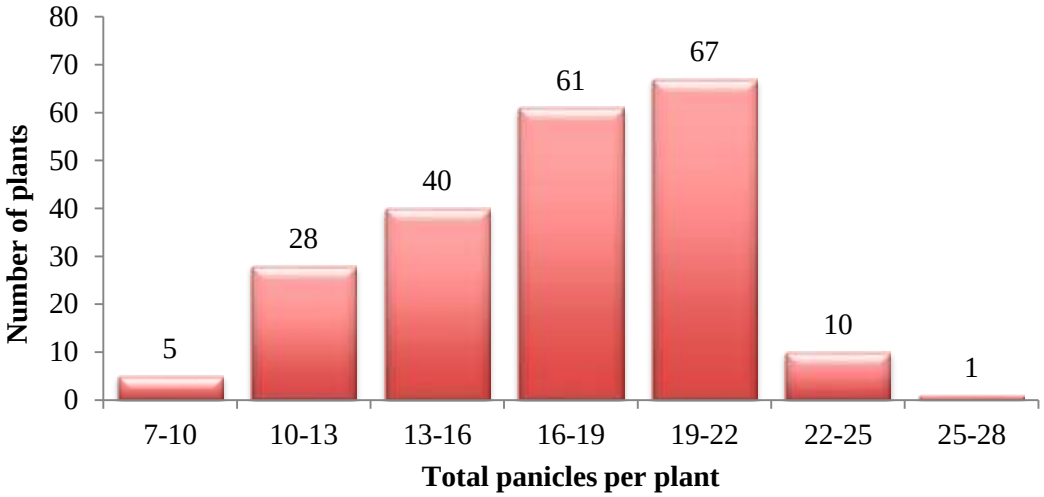


Fig. 4.2: Frequency distribution of total panicles per plant in rice germplasm

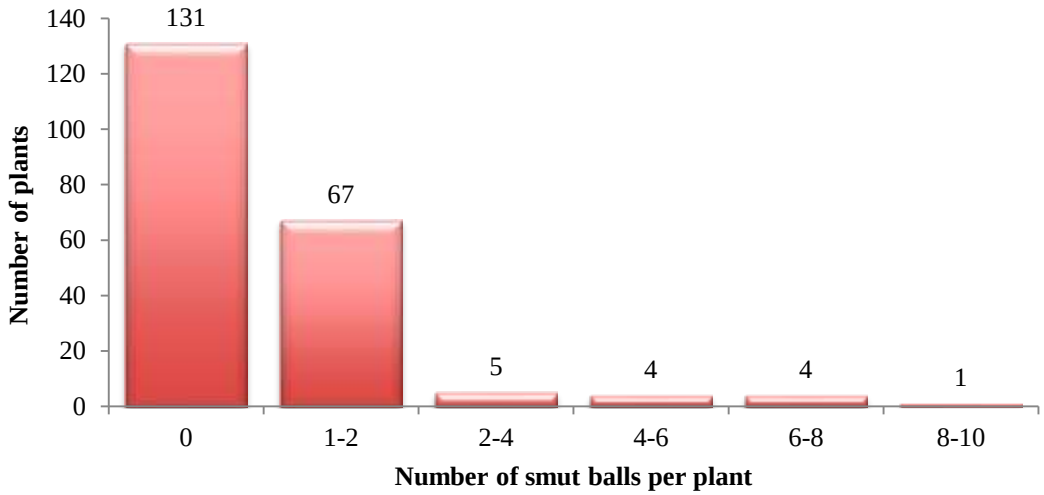


Fig. 4.3: Frequency distribution of number of smut balls per plant in rice germplasm

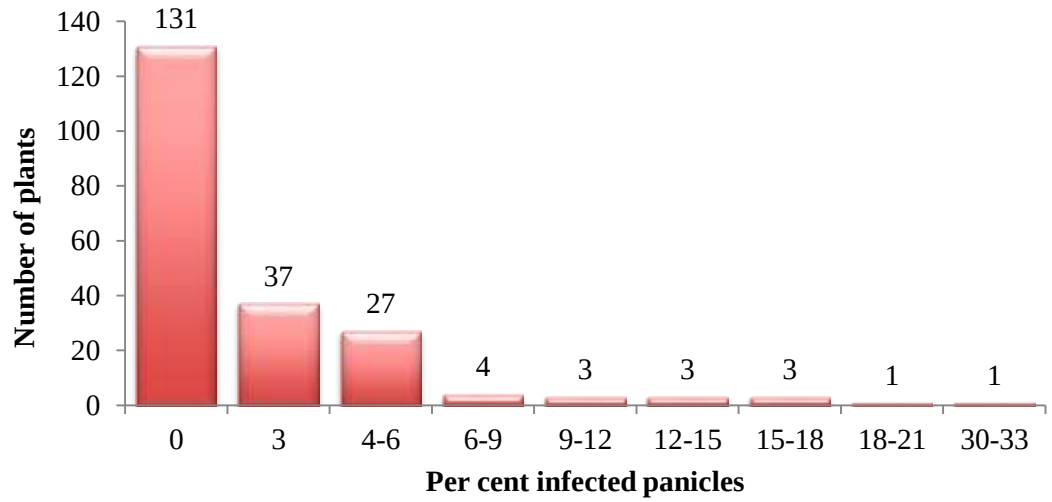


Fig. 4.4: Frequency distribution of per cent infected panicles in rice germplasm

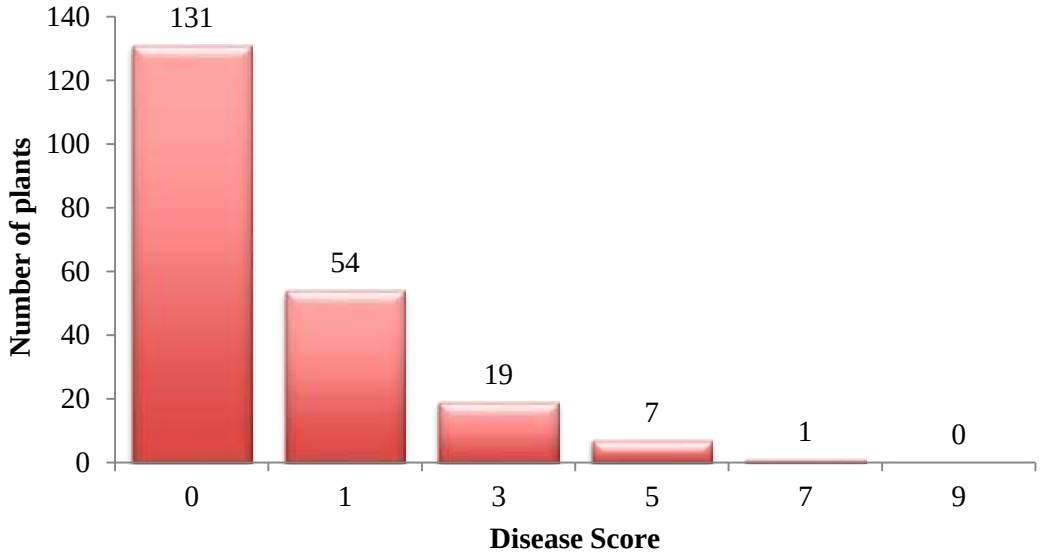


Fig. 4.5: Frequency distribution of disease score in rice germplasm

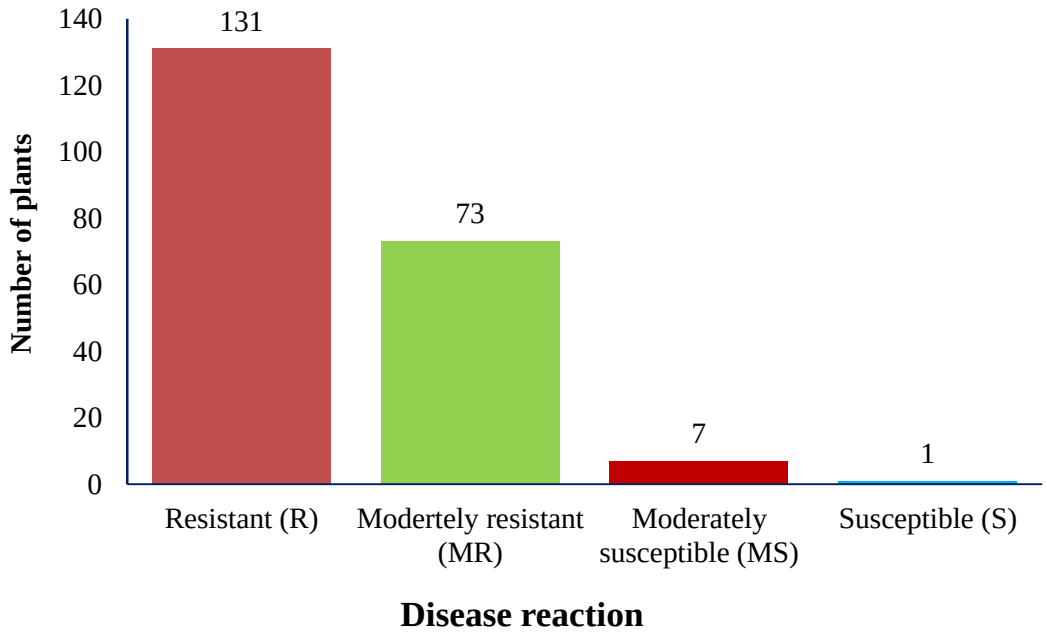


Fig. 4.6: Number of genotypes under different disease reaction group

The above data revealed a lot of variation in disease incidence of false smut among the 212 rice germplasm lines. The natural hotspots of disease allow us to screen our large breeding population along with artificial inoculation of selected lines would help us to identify resistant sources of rice against the false smut. A wide variation in the resistance of rice genotypes against the false smut of rice has been reported (Anand *et al* 1985, Singh *et al* 1987, Singh and Kang 1987, Singh and Khan 1989 and Sugha *et al* 1992, Kurauchi *et al* 2006, Lore *et al* 2013).

Biswas (2001) found eight rice hybrids completely free from the false smut disease under field conditions from 41 rice hybrids. Hedge (1998) screened 58 rice lines and selected 15 genotypes free from infection while remaining showed 2-20% infection by the pathogen.

Dodan and Singh (1996) evaluated 143 scented and non-scented rice varieties, only 20 of them exhibited false smut resistance. Similarly, Atia (2004) found Giza 171 to be highly susceptible to false smut as this is long duration variety in screening experiment of five commercial rice varieties.

Lore *et al* (2013) found quantitative variation in susceptibility level among rice genotypes against false smut of rice. After screening of 25 rice hybrids and 11 inbred lines, it was found that two hybrids NPH 909 and NPH 369 showed higher disease intensity while the hybrid cultivars, PR113 and PR114 exhibit lowest level of disease intensity.

Kaur *et al* (2015) evaluated 125 rice genotypes, found one inbred line and nine hybrids resistant to false smut disease. Rani *et al* (2016) evaluated 31 rice germplasm lines and 10 lines were found highly resistant against the disease based on the disease variables.

Banasode and Hosagoudar (2021) screened 102 rice genotypes under field conditions and found 11 genotypes were highly resistant and IR-64 was moderately resistant to disease on the basis of per cent infected panicles of rice. Hiremath *et al* (2021) found 25 rice lines were highly resistant in all the four years under natural as well as artificial conditions whereas 31 lines were moderately resistant to false smut after screening 125 rice accessions.

Table 4.4: Screening of rice germplasm lines (2K Panel, IRRI) under field conditions

S. No.	Designation	D 50% f	% IP	NSB	Max Score
1	CHIGYUNGDO::IRGC 55466-1	68	0	0	0
2	YAKUMO::IRGC 5320-1	72	0	0	0
3	ZHENSHAN 97 B	74	0	0	0
4	YAN ZHAO 9::IRGC 63062-1	74	0	0	0
5	CO 39::IRGC 51231-1	76	0	0	0
6	YANGKUM (RED)::IRGC 32406-1	91	0	0	0
7	AEDAL::IRGC 55441-1	74	0	0	0
8	GHASELU MAP::IRGC 72527-1	79	0	0	0
9	NIPPONBARE	79	0	0	0
10	DUMAI::IRGC 25852-1	79	0	0	0
11	KAM MRA::IRGC 62172-1	79	3.3	1	3

S. No.	Designation	D 50% f	% IP	NSB	Max Score
12	IR 73678-20-1-B::IRGC 117292-1	76	0	0	0
13	SADU CHO	76	3.8	0.6	1
14	M 202	74	1.7	0.4	1
15	BR IRGA 409::IRGC 116960-1	114	0	0	0
16	KUROKA::IRGC 74556-C1	85	0	0	0
17	IRAT 144::IRGC 55685-C1	82	0	0	0
18	WARABEHATOMOCHI::IRGC 14779-1	82	0	0	0
19	GOGO LEMPAK::IRGC 43392-C1	81	0	0	0
20	RIZZOTTO 264::IRGC 65727-1	82	0	0	0
21	WAB 368-B-1-H1-HB::IRGC 117359-1	82	0	0	0
22	DAWASAM (RED)::IRGC 32389-1	84	0	0	0
23	RIENALDO BERZANO::IRGC 3230-1	84	0	0	0
24	LI JIANG XIN TUAN HEI GU	87	0	0	0
25	DULAR	90	0	0	0
26	SHADA SHAITA::IRGC 64796-1	82	0	0	0
27	EDOGAWA::IRGC 74468-1	82	0	0	0
28	INDIO::IRGC 116998-1	83	0	0	0
29	CHAHORA 144::IRGC 27869-1	85	0	0	0
30	DA 28::IRGC 6246-1 (4246-1 ?)	87	0	0	0
31	INDANE::IRGC 33130-1	114	0	0	0
32	ARGO::IRGC 82418-1	83	0	0	0
33	BREVIARISTATA (RAIA)::IRGC 3189-1	86	0	0	0
34	KHUDWANI ACC 409::IRGC 34216-1	86	0	0	0
35	KATAKTARA DA 2::IRGC 32565-1	88	0	0	0
36	N 22	101	0	0	0
37	RP 1451-1196-1562-4218::IRGC 117351-1	88	31.3	28.8	7
38	NSICRC 106::IRGC 117370-1 (17370-1 ?)	88	8.9	5.2	5
39	PICONEGRO::IRGC 117022-1	82	0	0	0
40	GHARIB::IRGC 32306-1	83	0	0	0
41	ITA 235::IRGC 64854-1	92	0	0	0
42	DA 8::IRGC 6422-1	92	0	0	0
43	BRAZOS::IRGC 24273-1	92	0	0	0
44	CR 5272::IRGC 116971-1	99	0	0	0
45	EMBRAPA 6 CHUI::IRGC 116981-1	82	0	0	0

S. No.	Designation	D 50% f	% IP	NSB	Max Score
46	LATSIKA::IRGC 69367-1	82	0	0	0
47	JIMBRUK JOLOWORO::IRGC 43420-C1	88	0	0	0
48	BARAN BORO::IRGC 27509-1	95	0	0	0
49	KEN CHIAO JU HSIAO LI::IRGC 1217-1	95	0	0	0
50	1-52-6::IRGC 39111-1	95	0	0	0
51	BOSSA::IRGC 57781-1	95	5.9	1.6	3
52	LUDAN::IRGC 64189-C1	95	1.0	0.2	1
53	CIRAD 409::IRGC 116969-1	95	0	0	0
54	VANDANA::IRGC 117398-1	95	4.8	1.4	3
55	MINGHUI 63	95	0	0	0
56	CIMARRON::IRGC 116967-1	99	0	0	0
57	PALMIRA::IRGC 5097-1	104	2.8	0.4	1
58	IAC 164::IRGC 117251-1	104	3.7	0.8	1
59	DOONGARA::IRGC 78392-1	99	13.3	2	3
60	KETAN KONIR::IRGC 43444-C1	99	0	0	0
61	TRES MESES::IRGC 6464-C1	104	0	0	0
62	DA YE XI NAN::IRGC 73958-1	100	0	0	0
63	KURULU WEE (WHITE)::IRGC 66518-1	101	0	0	0
64	UCHUTI::IRGC 14694-1	101	2.2	0.4	1
65	RHS 107-2-1-2TB-1JM::IRGC 117025-1	101	1.7	0.2	1
66	MARS::IRGC 38541-1	101	1.3	0.2	1
67	SANHUANGZHAN NO 2	91	4.1	3	3
68	AZUCENA	101	0	1.2	3
69	GUATEMALA 1021::IRGC 3388-1	101	4.9	1.6	3
70	IAC 120::IRGC 22712-1	101	1.6	0.2	1
71	GUAYQUIRARO P A::IRGC 116987-1	101	0.9	0.2	1
72	TOS 10483::IRGC 56723-1	101	4.6	1	3
73	TOX 1011-4-1::IRGC 117033-1	101	3.0	0.4	1
74	TX 10438::IRGC 117035-1	101	7.5	1.2	3
75	BAKILIKINDA::IRGC 63121-1	101	2.5	0.6	1
76	PACHOLINHA::IRGC 50531-1	101	6.0	1.2	3
77	ORIENTE 10::IRGC 55808-1	101	4.3	0.8	1
78	MATAHAMBRE::IRGC 53200-1	107	2.4	0.4	1
79	CAIAPO::IRGC 116962-1	101	0	0	0

S. No.	Designation	D 50% f	% IP	NSB	Max Score
80	IC 27525::IRGC 53989-1	101	1.1	0.2	1
81	ARC 10317::IRGC 12430-1	102	1.1	0.2	1
82	GHARIB::IRGC 32303-1	102	5.1	0.8	1
83	AUS JOTA::IRGC 66767-1	102	1.5	0.2	1
84	BAKUNG (H)::IRGC 60220-1	102	9.9	6.6	5
85	JAMAICA 3::IRGC 4105-1	102	14.3	7.6	5
86	ARC 12701::IRGC 22267-1	102	1.6	0.2	1
87	MUT IAC 25-44-807::IRGC 68799-1	102	0	0	0
88	JI BO YA::IRGC 77446-1	102	13.3	4.8	3
89	CYPRESS	99	3.1	0.6	1
90	BICO BRANCO::IRGC 38994-1	99	3.0	0.8	1
91	CANA ROXA::IRGC 25966-1	102	0	0	0
92	KALAMKATI::IRGC 45975-1	102	3.2	1	3
93	MIKHUDEB::IRGC 25892-1	102	0	0	0
94	ARC 12451::IRGC 41052-1	99	1.9	0.8	1
95	KIKILONG::IRGC 71539-1	99	3.4	0.4	1
96	TOS 724::IRGC 11108-1	104	0	0	0
97	HAWM OM::IRGC 23729-1	104	0	0	0
98	IAC 1111::IRGC 39050-1	104	4.5	0.8	1
99	KHAO KAP SANG::IRGC 23423-1	104	0	0	0
100	EL PASO L 227::IRGC 116979-1	104	0	0	0
101	NATO::IRGC 1819-1	104	1.7	0.2	1
102	KHAO DAM::IRGC 23385-1	104	0	0	0
103	KINANDANG PATONG::IRGC 23364-1	104	0	0	0
104	MA HAE::IRGC 23754-1	104	0	0	0
105	RT 1031-69::IRGC 15092-1	104	2.9	0.4	1
106	TCHAMPA::IRGC 32362-1	100	1.2	0	0
107	HEI CHIAO CHUI LI HSIANG KENG::IRGC 1112-1	88	0	0	0
108	SAYLLEBON::IRGC 32509-1	100	0	0.4	1
109	MAINTIMOLOTSY 1226::IRGC 11010-C1	100	1.4	0.2	1
110	BAMAWPYAN::IRGC 72458-1	100	1.2	0.2	1
111	TAINUNG 67	100	0	0	0
112	DINOLORES::IRGC 67431-1	100	0	0	0

S. No.	Designation	D 50% f	% IP	NSB	Max Score
113	IR 64-21	100	0	0	0
114	DOMSIAH::IRGC 32292-1	100	0	0	0
115	BG 301::IRGC 117315-1	100	0	0	0
116	62-667::IRGC 15147-1	100	1.1	0.2	1
117	RXAR RGUE::IRGC 1943-1	100	0	0	0
118	63-104::IRGC 15100-C1	100	4.9	1	3
119	IA CUBA 23::IRGC 116991-1	100	0	0	0
120	DOM SUFID	100	1.4	0.2	1
121	VARY LAVA::IRGC 386-1	100	0	0	0
122	TORO 2::IRGC 66761-1	100	0	0	0
123	MALAGKIT PUTI::IRGC 19451-1	100	0	0	0
124	IR 65482-17-511-5-7::IRGC 117284-1	100	0	0	0
125	GOGO::IRGC 43390-C1	100	1.2	0.2	1
126	GSR105 (S)	100	11.5	4	3
127	GSR123 (S)	100	17.2	7.4	5
128	SAYARI::IRGC 74716-1	100	0	0	0
129	ARC 11495::IRGC 21431-1	100	1.2	0.2	1
130	IR 74371-3-1-1::IRGC 117373-1	100	0	0	0
131	IAC 47::IRGC 116992-1	100	0	0	0
132	TOANG::IRGC 19144-1	102	1.1	0.2	1
133	TAINUNG 29::IRGC 65309-1	102	0	0	0
134	TOS 5790::IRGC 117256-1	102	3.1	0.4	1
135	IR 77298-14-1-2::IRGC 117374-1	102	0	0	0
136	JHONA 26::IRGC 27967-1	102	0	0	0
137	CCT 3-37-3-3-3-1::IRGC 117322-1	108	0	0	0
138	KHAO DO NGOI::IRGC 29772-1	108	0	0	0
139	PULU LAPA::IRGC 48857-C1	104	3.2	1	1
140	LAMBAYQUE 1::IRGC 10769-1	108	0	0	0
141	TCHAMPA::IRGC 32368-1	103	0	0	0
142	BAGANAN ASALAO::IRGC 71503-1	108	0	0	0
143	SILADON::IRGC 71620-1	103	4.8	0.2	1
144	PERLA::IRGC 117021-1	107	4.8	1.4	3
145	CAPI 93::IRGC 116964-1	107	20.2	7.2	5
146	GANIGI::IRGC 48698-C1	104	0	0	0

S. No.	Designation	D 50% f	% IP	NSB	Max Score
147	PADI KOMPAI::IRGC 25510-1	97	0	0	0
148	OS 4::IRGC 11335-C1	97	0	0	0
149	MIMIDAM::IRGC 25897-1	110	0	0	0
150	CT 6946-9-1-2-M-1P::IRGC 117329-1	92	0	0	0
151	C 8434::IRGC 13496-1	104	0	0	0
152	IR 72132-AC 6-1::IRGC 117369-1	106	1.4	0.2	1
153	ICTA POLOCHIC::IRGC 116997-1	105	1.3	0.2	1
154	INIAP 415::IRGC 117001-1	107	0	0	0
155	IR 60080-46 A::IRGC 117396-1	103	0	0	0
156	BENGIZA::IRGC 69845-1	103	0	0	0
157	VILLAGUAY P A::IRGC 117259-1	103	0	0	0
158	NS 1288::IRGC 68930-1	103	0	0	0
159	B 6144 F-MR-6::IRGC 117313-1	103	1.2	0.2	1
160	PITIPO::IRGC 117023-1	111	4.8	1.4	3
161	MAHAPLEU (504)::IRGC 50865-1	108	0	0	0
162	CT 9993-5-10-1-M::IRGC 116974-1	108	0	0	0
163	D 4-136::IRGC 31051-1	108	0	0	0
164	RANAU KADAI::IRGC 71604-1	108	3.4	0.4	1
165	J 104::IRGC 117008-1	108	0	0	0
166	ARC 11294::IRGC 21296-1	108	0	0	0
167	GOMPA 2::IRGC 12894-1	108	0	0	0
168	DAKPA::IRGC 64888-1	108	0	0	0
169	FOSSA HV::IRGC 16069-1	108	0	0	0
170	VASSE NANAN::IRGC 56812-1	111	1.6	0.2	1
171	MOROBEREKAN	111	1.5	0.2	1
172	NILO 3 B::IRGC 10283-1	109	8.6	2	3
173	YANCAOUSSA::IRGC 16071-C1	105	2.9	0.4	1
174	GBANTE::IRGC 16081-1	108	4.6	0.8	1
175	GAO GAN DA NUO::IRGC 73974-1	107	0	0	0
176	KAKANI 2::IRGC 13373-C1	113	0	0	0
177	JUMA 51::IRGC 117009-1	116	0	0	0
178	TAK SIAH::IRGC 73126-1	109	0	0	0
179	PAE URA::IRGC 27321-1	116	0	0	0
180	SPR 87032-2-1-1-4::IRGC 117355-1	110	1	0.6	1

S. No.	Designation	D 50% f	% IP	NSB	Max Score
181	ONDEYKAM::IRGC 67846-1	110	0	0	0
182	IR 43::IRGC 117005-1	110	0	0.2	1
183	IR 77384-12-35-3-12-1-B::IRGC 117299-1	116	3.6	0.4	1
184	NHTA 5::IRGC 186-1	106	0	0	0
185	ICTA PAZOS::IRGC 116996-1	114	0	0	0
186	GEMJYA JYANAM::IRGC 32411-C1	106	0	0	0
187	DOM ZARD::IRGC 12881-1 (1288-1 ?)	116	0	0	0
188	RATHAL::IRGC 31525-1	116	1.9	0.2	1
189	EPAGRI 109::IRGC 116983-1	116	0	0	0
190	CINA::IRGC 27116-1	113	0	0	0
191	BATUKURU WEE::IRGC 67614-1	119	0	0	0
192	DAVAO::IRGC 8244-C1	119	0	0	0
193	REXORO::IRGC 1715-1	102	0	0	0
194	ABRI::IRGC 32380-1	127	0	0	0
195	WALANGA::IRGC 27502-1	117	1.3	0.8	1
196	PANAMA 1537::IRGC 117019-1	125	0	0	0
197	IR 80314-4-B-1-3-B::IRGC 117308-1	130	1.5	0	1
198	NENG NAH::IRGC 78275-1	116	0	0	0
199	OIRAN::IRGC 8257-1	116	0	0	0
200	RATHAL::IRGC 31524-1	119	3.8	0.4	1
201	NAVOLATO A 71::IRGC 117016-1	127	5.8	1.4	3
202	KHAU MEO::IRGC 78330-1	119	0	0	0
203	NEP ME HOA BINH::IRGC 78366-1	127	0	0	0
204	JUMALI::IRGC 9542-C1	130	0	0	0
205	SAMBA MAHSURI::IRGC 117377-1	125	0	0	0
206	KHAO' MUM::IRGC 78259-1	125	1.4	0.2	1
207	CR 8334::IRGC 116972-1	130	0	0	0
208	PTB 25::IRGC 6386-1	120	0	0	0
209	RYT3229 (PAU3835-12-1-1-2) (Sus)	112	2.4	0.4	1
210	PR116 (S)	112	9.6	3.2	3
211	GSR105 (S)	100	16.5	12.6	5
212	GSR123 (S)	100	17.9	11.2	5

D 50% f: Days to 50% flowering; % IP: Per cent infected panicle; NSB: Number of smut balls; Max Score: Maximum disease score

4.5 Screening of selected resistant rice germplasm accessions for identification of potential donors imparting resistance to false smut

The natural hotspots of disease allow us to screen our large breeding population along with artificial inoculation of selected lines would help us to identify resistant sources of rice against the false smut. The screening of 212 rice germplasm lines (Table 4.4) was done under field conditions naturally depending upon various environmental conditions for the incidence of this disease. The lines which were completely free from infection by the false smut and have certain good agronomic characters were chosen based on disease score (Table 4.5). The main objective of selection was identification of potential donors imparting resistant to false smut in rice which can be used further in breeding programs for transferring resistance to false smut in otherwise susceptible commercial varieties of rice.

Out of 212 germplasm lines, 27 resistant lines with good agronomic traits were selected after screening under field conditions based on 2017-20 screening data of disease score (Table 4.5). These 27 lines were screened along with susceptible checks (GSR123 and PR116) under field conditions in 2017 (Table 4.6). Among 27 screened resistant lines, 19 lines were found completely free from the disease under field conditions with disease score of zero while the lines with S.No. 1, 3, 4, 5, 6, 9, 11, 13 and 18 were reported with disease score of 1 in one or more years of screening (Table 4.6). On the other hand, disease score of 9 and 7 were observed on GSR123 and PR116 respectively. The per cent infected panicle of GSR123 and PR116 were 54.9% and 39.0% respectively (Table 4.7). The highest infected panicle was observed in BG301 (25%) (Table 4.7). The screening of selected resistant lines was also done under artificial conditions.

4.6 Screening of selected resistant rice germplasm accessions under artificial conditions for identification of potential donors imparting resistance to false smut

The selected 27 resistant germplasm lines along with two standard checks (GSR123 and PR116) were also screened under controlled conditions. The data on various disease variables including total panicles, total infected panicles, number of smut balls per panicle and per cent infected panicle was recorded (Table 4.7). All of the 27 tested lines under controlled conditions were found to be completely free from the disease with disease score of 0 except the two susceptible checks (GSR123 and PR116) on which disease score of 9 and 7 was observed. The per cent infected panicles in GSR 123 were recorded 45% whereas in PR116 recorded 28% infected panicles (Table 4.7). The number of smut balls per panicle in GSR123 were 5.5 as compared to 3.0 in PR116.

The screening of rice germplasm lines against this disease is done through artificial inoculation by boot injection method. The boot stage of rice or flowering stage is the most susceptible stage for infection by this pathogen (Biswas 2001, Ashizawa and Kataoka 2005,

Table 4.5: Screening of selected resistant rice accessions as potential donors for breeding program under field conditions over years (2017-20)

S.No.	Designation	IRGC No.	sub pop	2017	2018	2019	2020
1	BARAN BORO::IRGC 27509-1	117434	aus	0	0	0	1
2	KEN CHIAO JU HSIAO LI::IRGC 1217-1	117512	temperate-japonica	0	0	0	0
3	CR 5272::IRGC 116971-1	120918	indica	0	1	1	0
4	BG 301::IRGC 117315-1	120892	indica	1	1	1	1
5	VARY LAVA::IRGC 386-1	121685	tropical-japonica	0	1	0	0
6	TORO 2::IRGC 66761-1	124468	tropical-japonica	0	1	0	0
7	IAC 47::IRGC 116992-1	121361	tropical-japonica	0	0	0	0
8	DA YE XI NAN::IRGC 73958-1	124475	NA	0	0	0	0
9	DOMSIAH::IRGC 32292-1	117467	NA	0	1	0	0
10	TAINUNG 29::IRGC 65309-1	121644	temperate-japonica	0	0	0	0
11	MUT IAC 25-44-807::IRGC 68799-1	121672	tropical-japonica	0	1	0	0
12	CANA ROXA::IRGC 25966-1	117440	tropical-japonica	0	0	0	0
13	IR 60080-46 A::IRGC 117396-1	117596	tropical-japonica	0	1	0	0
14	HAWM OM::IRGC 23729-1	117484	admixed-japonica	0	0	0	0
15	GAO GAN DA NUO::IRGC 73974-1	121340	temperate-japonica	0	0	0	0
16	DAKPA::IRGC 64888-1	121563	admixed	0	0	0	0
17	TCHAMPA::IRGC 32362-1		aus	0	0	0	0
18	HEI CHIAO CHUI LI HSIANG KENG::IRGC 1112-1	121690	admixed	0	0	0	1
19	ITA 235::IRGC 64854-1	121368	tropical-japonica	0	0	0	0
20	PADI KOMPAI::IRGC 25510-1 TJ	124490	tropical-japonica	0	0	0	0
21	REXORO::IRGC 1715-1 TJ	117568	tropical-japonica	0	0	0	0
22	GEMJYA JYANAM::IRGC 32411-C1	121739	admixed Japonica	0	0	0	0
23	ARC 11294::IRGC 21296-1	121658	tropical-japonica	0	0	0	0
24	KHAO DO NGOI::IRGC 29772-1	121208	admixed Japonica	0	0	0	0
25	TAK SIAH::IRGC 73126-1	121224	admixed	0	0	0	0
26	KAKANI 2::IRGC 13373-C1	122128	admixed	0	0	0	0
27	INDANE::IRGC 33130-1	117492	admixed Japonica	0	0	0	0
28	GSR123 (S)			7	7	7	5
29	PR116 (S)			7	7	5	7

The shaded rows ranging S.No. 28-29 are the susceptible checks used under this study

Table 4.6: Screening of selected resistant rice accessions as potential donors for breeding program under field conditions in 2017

Sr. No.	Designation	IRGC No.	DF 50%	%IP	NSB/Panicle	NSB/Plant	Score
1	BARAN BORO::IRGC 27509-1	117434	95	0	0	0	0
2	KEN CHIAO JU HSIAO LI::IRGC 1217-1	117512	95	0	0	0	0
3	CR 5272::IRGC 116971-1	120918	99	19.0	1	2	1
4	BG 301::IRGC 117315-1	120892	100	25.0	2	4	5
5	VARY LAVA::IRGC 386-1	121685	100	24.2	1	2	1
6	TORO 2::IRGC 66761-1	124468	100	12.5	5	5	3
7	IAC 47::IRGC 116992-1	121361	100	19.0	3	3	1
8	DA YE XI NAN::IRGC 73958-1	124475	100	0	0	0	0
9	DOMSIAH::IRGC 32292-1	117467	100	9.1	3	3	5
10	TAINUNG 29::IRGC 65309-1	121644	102	0	0	0	0
11	MUT IAC 25-44-807::IRGC 68799-1	121672	102	21.4	1	1	5
12	CANA ROXA::IRGC 25966-1	117440	102	0.0	0	0	0
13	IR 60080-46 A::IRGC 117396-1	117596	103	5.6	3	3	3
14	HAWM OM::IRGC 23729-1	117484	104	0	0	0	0
15	GAO GAN DA NUO::IRGC 73974-1	121340	107	0	0	0	0
16	DAKPA::IRGC 64888-1	121563	108	0	0	0	0
17	TCHAMPA::IRGC 32362-1	117585	100	0	0	0	0
18	HEI CHIAO CHUI LI HSIANG KENG::IRGC 1112-1	121690	100	0	0	0	0
19	ITA 235::IRGC 64854-1	121368	92	0	0	0	0
20	PADI KOMPAI::IRGC 25510-1 TJ	124490	97	0	0	0	0
21	REXORO::IRGC 1715-1 TJ	117568	102	0	0	0	0
22	GEMJYA JYANAM::IRGC 32411-C1	121739	106	0	0	0	0
23	ARC 11294::IRGC 21296-1	121658	108	0	0	0	0
24	KHAO DO NGOI::IRGC 29772-1	121208	108	0	0	0	0
25	TAK SIAH::IRGC 73126-1	121224	109	0	0	0	0
26	KAKANI 2::IRGC 13373-C1	122128	113	0	0	0	0
27	INDANE::IRGC 33130-1	117492	114	0	0	0	0
28	GSR123 (S)		100	54.9	5.5	22	9
29	PR116 (S)		112	39.0	3	16	7

The shaded rows ranging S.No. 28-29 are the susceptible checks used under this study

DF 50%: Days to 50% flowering; % IP: Per cent infected panicle; NSB: Number of smut balls; Score: Disease score

Table 4.7: Screening of selected rice accessions as potential donors for breeding program under artificial conditions

S.No.	Designation	IRGC No.	%IP	NSB/P	NSB/Plant	Score
1	BARAN BORO::IRGC 27509-1	117434	0	0	0	0
2	KEN CHIAO JU HSIAO LI::IRGC 1217-1	117512	0	0	0	0
3	CR 5272::IRGC 116971-1	120918	0	0	0	0
4	BG 301::IRGC 117315-1	120892	0	0	0	0
5	VARY LAVA::IRGC 386-1	121685	0	0	0	0
6	TORO 2::IRGC 66761-1	124468	0	0	0	0
7	IAC 47::IRGC 116992-1	121361	0	0	0	0
8	DA YE XI NAN::IRGC 73958-1	124475	0	0	0	0
9	DOMSIAH::IRGC 32292-1	117467	0	0	0	0
10	TAINUNG 29::IRGC 65309-1	121644	0	0	0	0
11	MUT IAC 25-44-807::IRGC 68799-1	121672	0	0	0	0
12	CANA ROXA::IRGC 25966-1	117440	0	0	0	0
13	IR 60080-46 A::IRGC 117396-1	117596	0	0	0	0
14	HAWM OM::IRGC 23729-1	117484	0	0	0	0
15	GAO GAN DA NUO::IRGC 73974-1	121340	0	0	0	0
16	DAKPA::IRGC 64888-1	121563	0	0	0	0
17	TCHAMPA::IRGC 32362-1	117585	0	0	0	0
18	HEI CHIAO CHUI LI HSIANG KENG::IRGC 1112-1	121690	0	0	0	0
19	ITA 235::IRGC 64854-1		0	0	0	0
20	PADI KOMPAI::IRGC 25510-1 TJ		0	0	0	0
21	REXORO::IRGC 1715-1 TJ		0	0	0	0
22	GEMJYA JYANAM::IRGC 32411-C1		0	0	0	0
23	ARC 11294::IRGC 21296-1	121658	0	0	0	0
24	KHAO DO NGOI::IRGC 29772-1	121208	0	0	0	0
25	TAK SIAH::IRGC 73126-1	121224	0	0	0	0
26	KAKANI 2::IRGC 13373-C1	122128	0	0	0	0
27	INDANE::IRGC 33130-1	117492	0	0	0	0
28	GSR123 (S)		45	5.5	22	9
29	PR116 (S)		28	3	16	7

The shaded rows ranging S.No. 28-29 are the susceptible checks used under this study

DF 50%: Days to 50% flowering; % IP: Per cent infected panicle; NSB: Number of smut balls; Score: Disease score

Ou, 1985, Schroud and TeBeest, 2005, Zhou *et al* 2003, Zhou *et al* 2008). Various researchers injected spore suspension into the leaf sheath at booting stage of rice for artificial inoculation of pathogen (Yoshino and Yamamoto 1952, Ikegami 1960, Fujita *et al* 1989, Ashizawa *et al* 2011, Ladhalakshmi *et al* 2012, Hu *et al* 2021).

The most widely accepted and effective method of inoculation was developed by Fujita *et al* (1989). The conidial suspension (1×10^6 conidia/ml) of 2ml was used for injection into leaf sheath of rice at booting stage. Ladhalakshmi *et al* (2012) artificially inoulated TN-1 under greenhouse conditions by injecting rice plants with 2ml of conidial suspension (2.5×10^5 conidia/ml) of *U. virens* at boot stage. Haiyong *et al* (2015) tested pathogenicity of *U. virens* isolates on six rice varieties using boot injection method.

4.7 Quantitative trait loci mapping for resistance to false in rice

Based on screening under field and artificial conditions, two lines namely CANAROXA and IAC 47 (Plate 9) were selected as resistant parents to identify QTLs for resistance to false smut. CANAROXA is a short duration, tropical japonica rice line originating from Brazil. It takes about 102 days to flower and based on disease score of 0 it was grouped as resistant parent. This rice accession was found to be resistant in all the four years of screening under field conditions (Table 4.5) as well as under artificial condition (Table 4.7). IAC47 is another tropical japonica rice line originated from Brazil and was also found to be resistant in all the four years of screening under field conditions as well as under artificial condition. Its average height is 200 cm and takes about 100 days to flower. The two selected resistant lines were crossed with two susceptible rice varieties, PR116 and PR126 to generate mapping populations. PR116 is a popular commercial cultivar of rice which was sown on a wide scale under Punjab state however is highly susceptible to false smut while PR126 is commercial cultivar being grown on wide scale, but again susceptible to false smut.

4.7.1 Phenotyping of F_2 mapping population

About 300 plants of F_2 population of both the crosses (PR126 × CANAROXA and PR116 × IAC 47) were screened for resistance to false smut under field conditions in year. The incidence of disease was very less in that year due to which it was difficult to identify resistant plants from the population. The environmental conditions during the most susceptible stage of plants in population plays major role in disease development in spite of pathogen load during that time. The high temperature and low relative humidity at that time was not conducive for *U. virens* to establish pathogenic relationship with plants.

However, few plants of both the F_2 populations showed susceptible reaction under lower disease incidence as well. These plants could have shown highly susceptible reaction under favourable weather conditions. Most of the susceptible F_2 plants of the cross between PR126 × CANAROXA exhibited disease score of 5 based on the percentage of infected

panicles (Table 4.8). Out of 23 susceptible plants, the maximum disease score of 7 was observed in three plants. The disease score of 5 was recorded on PR126, otherwise highly susceptible cultivar to false smut (Plate 10).

Table 4.8: Plants from F_2 population of cross PR126 × CANAROXA along with susceptible parent showing susceptible reaction

PR126⨯CANAROXA	%IP	NSB/P	NSB/Plant	Score
1	12.5	1	1	5
2	10.0	3	3	5
3	16.7	1	1	5
4	22.2	8	16	5
5	42.9	4	11	7
6	12.5	6	6	5
7	10.0	1	1	5
8	50.0	1	4	7
9	8.3	10	10	5
10	10.0	2	2	5
11	14.3	1	1	5
12	22.2	1.5	3	5
13	9.1	1	1	5
14	11.1	1	1	5
15	10.0	2	2	5
16	16.7	1	1	5
17	14.3	2	2	5
18	11.1	1	1	5
19	14.3	1	1	5
20	11.1	1	1	5
21	12.5	1	1	5
22	11.1	1	1	5
23	28.6	2	4	7
PR126	25.0	1	2	5
CANA ROXA	0.0	0	0	0

%IP (Percent infected panicles), Score (Disease score)
NSB/P (Number of smut balls per panicle),
NSB/Plant (Number of smut balls per plant)

Plate 9: The selected resistant donor parent in the crosses prepared under current study.

Plate 10: The morphology characteristics of the cross PR126 × CANAROXA (a) Recipient parent PR126 (b) F_1 progeny (c) Donor parent CANAROXA.

On the other hand, the maximum disease score of 9 (Table 4.9) was observed on PR116 which is used as a female parent in cross between PR116 × IAC 47. Most of the susceptible F_2 plants of the cross between PR116 × IAC 47 exhibited disease score of 5 based on the percentage of infected panicles and out of 23 susceptible plants, the maximum disease score of 7 was observed (Table 4.9). The resistant plants were selected randomly from the entire population based on the complete absence of disease on the plants.

Table 4.9: Plants from F_2 population of cross PR116 × IAC 47 along with susceptible parent showing susceptible reaction

PR116×IAC 47	%IP	NSB/P	NSB/Plant	Score
1	16.7	1	1	5
2	12.5	1	1	5
3	11.1	1	1	5
4	14.3	4	4	5
5	16.7	4	4	5
6	14.3	1	1	5
7	33.3	1.5	3	7
8	12.5	6	6	5
9	40.0	1	2	7
10	33.3	3.8	15	7
11	14.3	2	2	5
12	11.1	1	1	5
13	10.0	1	1	5
14	12.5	1	1	5
15	16.7	1	1	5
16	14.3	1	1	5
17	14.3	1	1	5
18	11.1	1	1	5
19	12.5	1	1	5
20	10.0	1	1	5
21	16.7	1	1	5
22	11.1	1	1	5
23	20.0	1	1	5
PR116	62.5	3.4	17	9
IAC 47	0.0	0	0	0

%IP (Percent infected panicles), Score (Disease score)
NSB/P (Number of smut balls per panicle),
NSB/Plant (Number of smut balls per plant)

The natural hotspots for false smut of rice will be helpful for phenotyping plants and their respective categorization under susceptible and resistant category. The population analysis for false smut disease under natural hotspot conditions and artificial inoculation conditions will help to identify QTL for imparting resistance to false smut.

4.7.2 Genotyping and identification of marker-trait association

Our objective was to identify QTLs for resistant to false smut based on genotyping and phenotyping of F_2 mapping populations. The aim could have been accomplished by identification of truly resistant and susceptible plants to false smut from both the populations and performing bulked segregant analysis (Michelmore *et al* 1991) to identify DNA regions associated with resistance to false smut. However due to less incidence of false smut during the phenotying year, the resistant plants could not be identified. However few susceptible plants from both the populations were identified and while resistant plants were selected at random from both the populations. DNA of selected susceptible and resistant plants along with parents were extracted and amplified with a set of SSR markers. The modified aim was to identify association of any marker with susceptible plants along with susceptible parents based on idea that any marker which is associated, will show similar band size in all the susceptible plants and susceptible parents, while resistant parent will show polymorphism and random resistant plants may or may not show the same band of susceptible plants and susceptible parent.

A total of seventy-eight SSR markers distributed on all of the 12 chromosomes from the universal core genetic map of rice (Orjuela *et al* 2010) were used for detecting polymorphism between the parents. SSRs were scored visually on the basis of difference in band size in each of the two parents and F_2 plants (Plate 11-14). The allelic data of polymorphic SSR markers were used to access the variation in the selected resistant and susceptible plants from mapping (Plate 15-23). Out of 78 SSR markers, only 12 markers showed polymorphism, while 31 were monomorphic and others could not be amplified (Table 4.10).

The markers which showed polymorphism in parents were RM104, RM7434, RM18457, RM289, RM16493, RM7434, RM8243, RM264, RM25149, RM10, RM26643 and RM8404 (Table 4.10, Plate 11-14). Among these, the marker RM25149 was found to be polymorphic (Plate 17 and 20) in parents of both the crosses (PR126 × CANAROXA and PR116 × IAC 47). The polymorphic markers in case of PR126 × CANAROXA includes RM104, RM289, RM7434, RM8243, RM264, RM25149, RM10 and RM26643 on chromosome 1, 5, 6, 8, 10 and 11, respectively (Table 4.11, Plate 14-19). The markers which showed polymorphism in PR116 × IAC 47 includes RM16493, RM18457, RM25149 and RM28404 on chromosome 4, 5, 10 and 12, respectively (Table 4.12, Plate 20-23). These

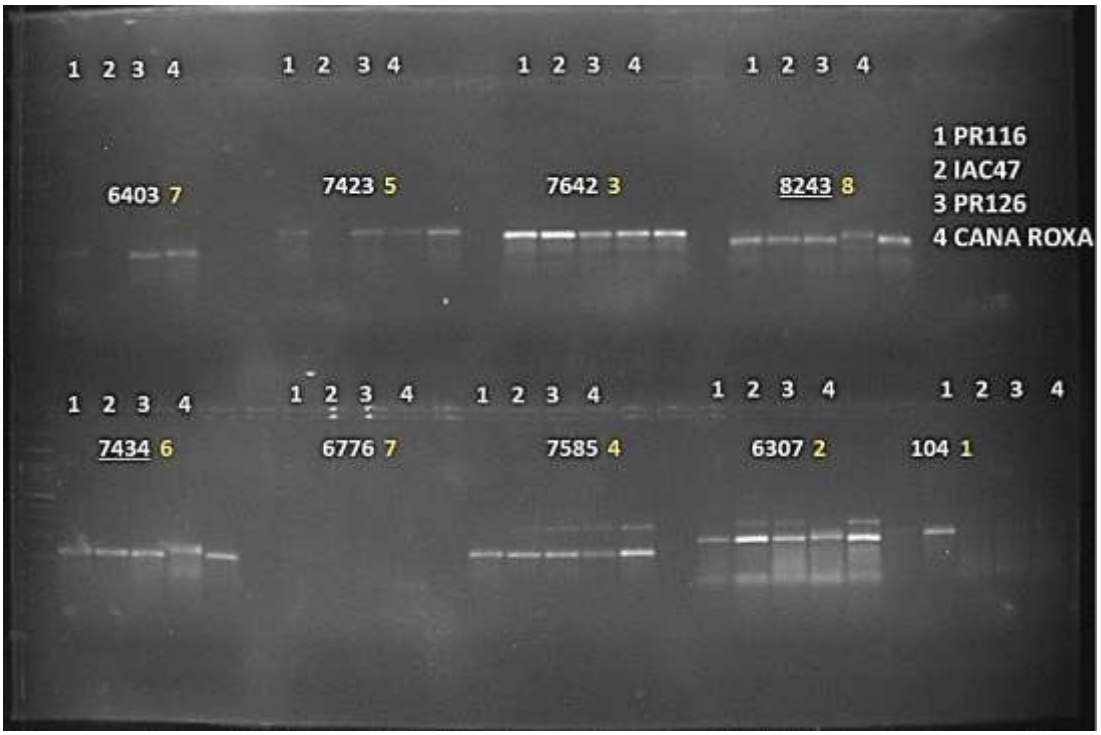


Plate 11: PCR based amplification of SSR markers on parents: 1) PR116, 2) IAC47, 3) PR126 and 4) CANAROXA.

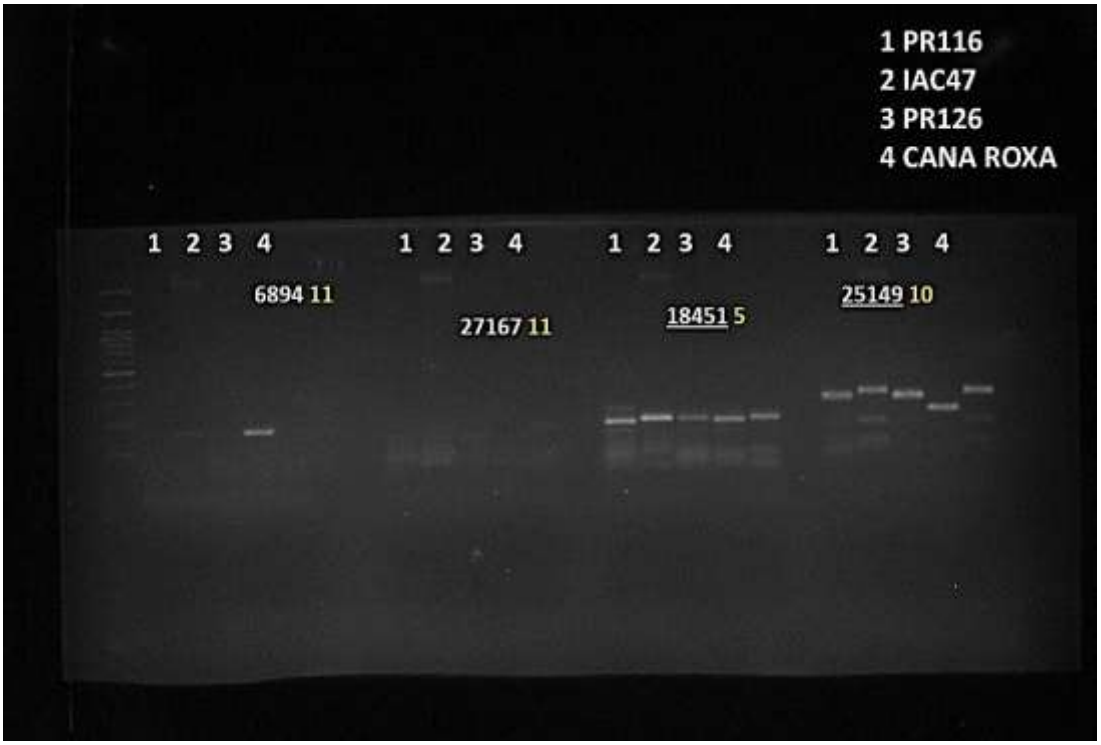


Plate 12: PCR based amplification of SSR markers on parents: 1) PR116, 2) IAC47, 3) PR126 and 4) CANAROXA.

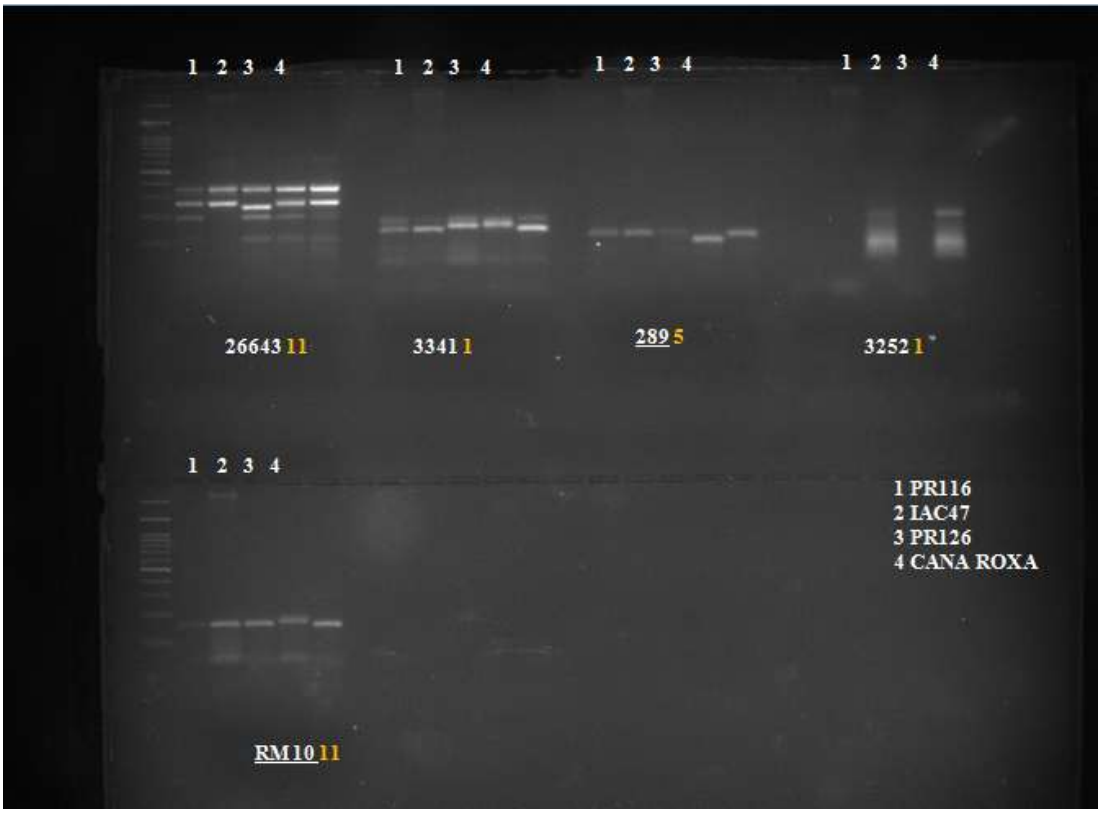


Plate 13: PCR based amplification of SSR markers on parents: 1) PR116, 2) IAC47, 3) PR126 and 4) CANAROXA.

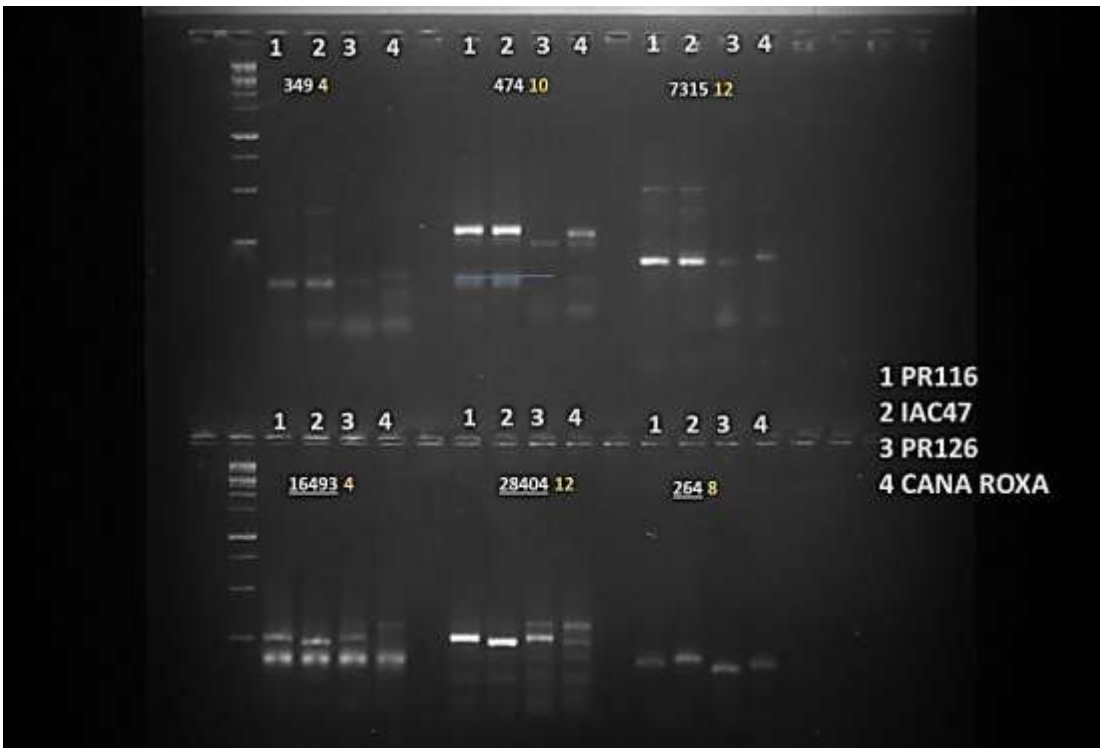


Plate 14: PCR based amplification of SSR markers on parents: 1) PR116, 2) IAC47, 3) PR126 and 4) CANAROXA.

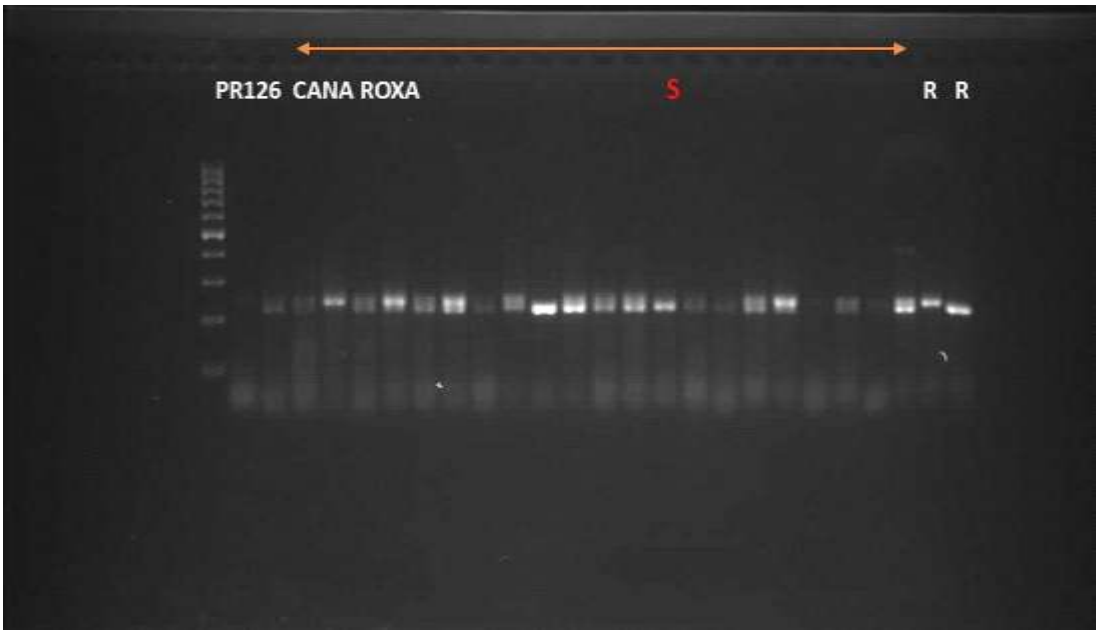


Plate 15: PCR based amplification of SSR marker RM104 (chromosome 1) on parents PR126 × CANAROXA and mapping population. 'S' denotes susceptible plants while 'R' denotes resistant plants of mapping population.

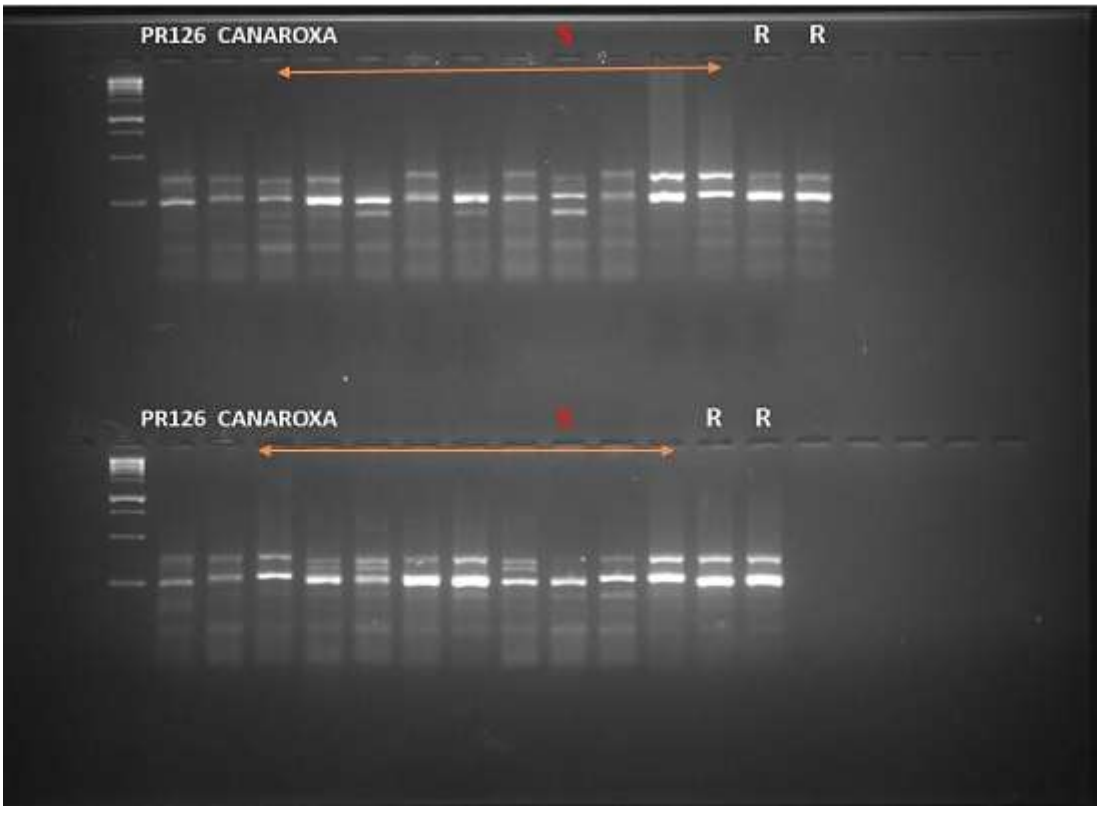


Plate 16: PCR based amplification of SSR marker RM26643 (chromosome 11) on parents PR126 and CANAROXA and PR126 × CANAROXA population. 'S' denotes susceptible plants while 'R' denotes resistant plants of mapping population.

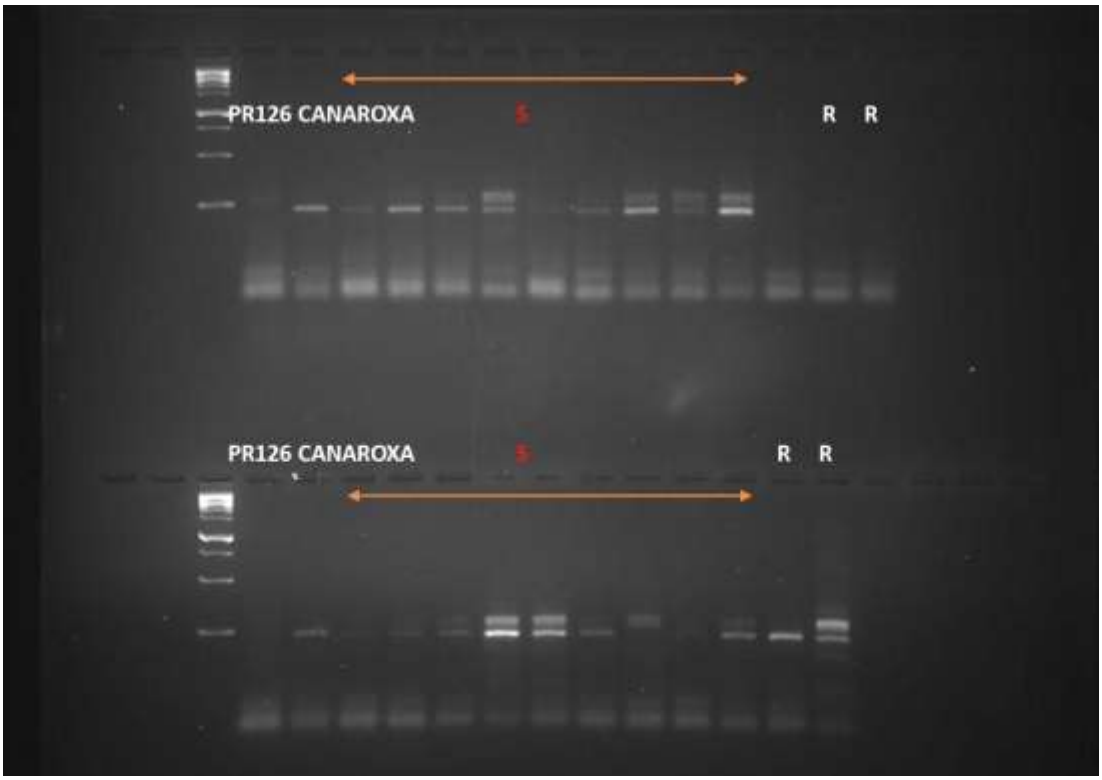


Plate 17: PCR based amplification of SSR marker RM25149 (chromosome 10) on parents PR126 × CANAROXA and mapping population. 'S' denotes susceptible plants while 'R' denotes resistant plants of mapping population.

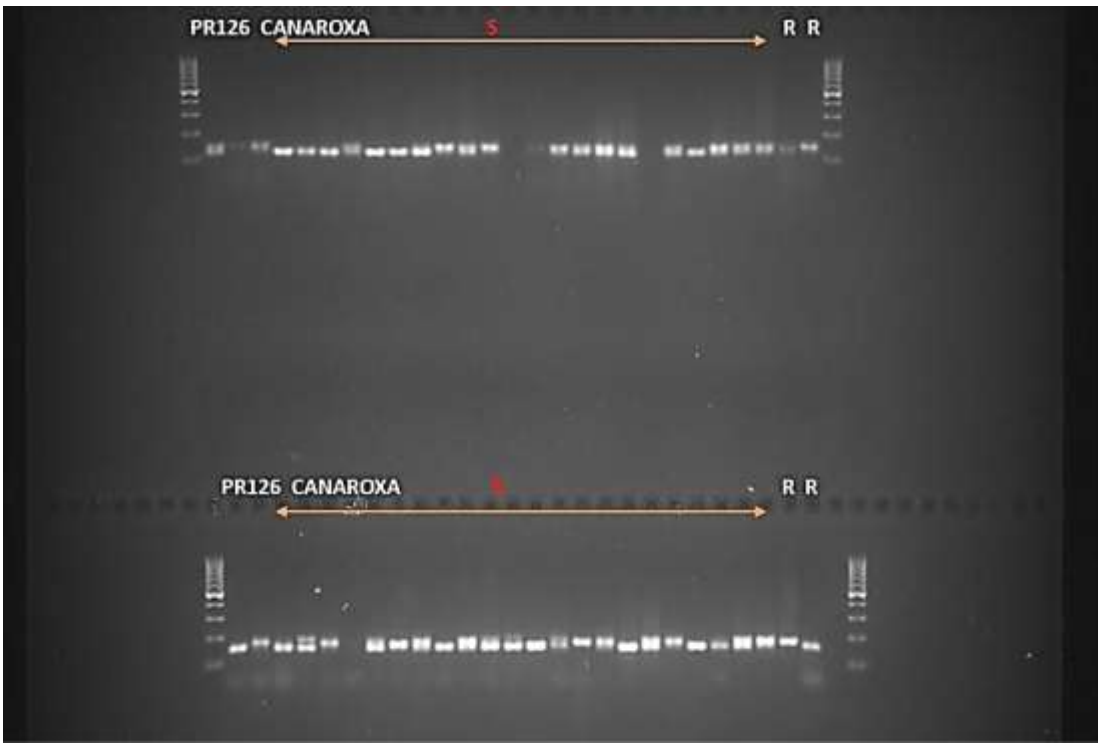


Plate 18: PCR based amplification of SSR marker RM7434 (top) and RM 8243 (bottom, chromosome 6 and 8 respectively) on parents PR126 × CANAROXA and mapping population. 'S' denotes susceptible plants while 'R' denotes resistant plants of mapping population.

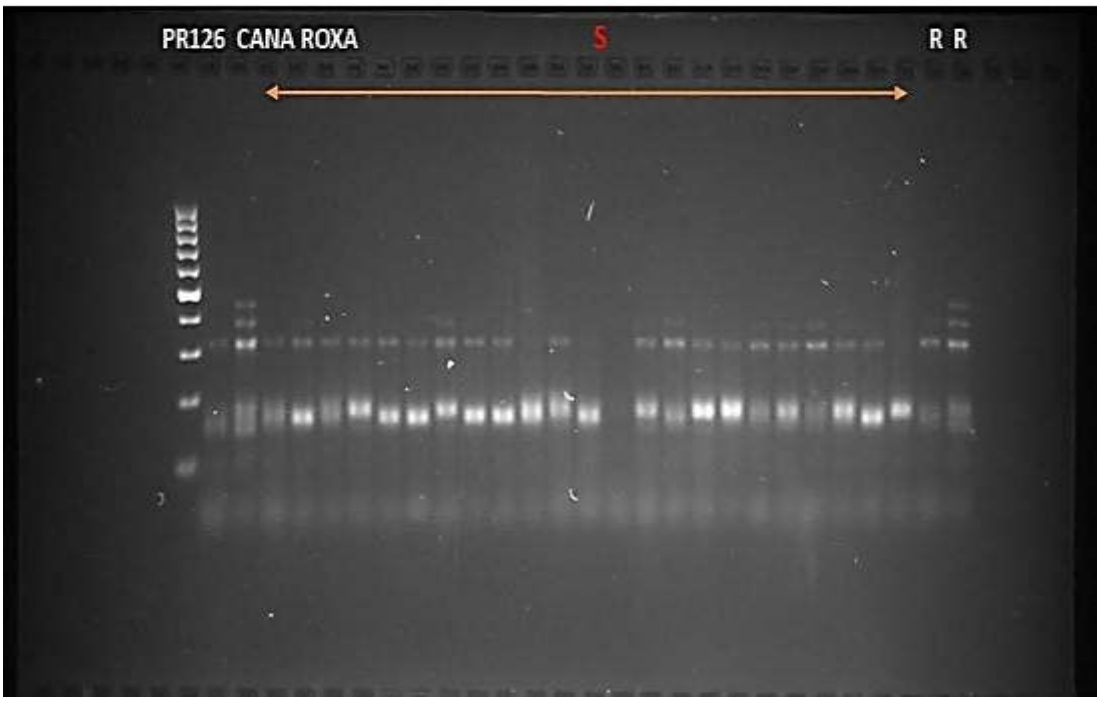


Plate 19: PCR based amplification of SSR marker RM10 (chromosome 11) on parents PR126 × CANAROXA and mapping population. 'S' denotes susceptible plants while 'R' denotes resistant plants of mapping population.

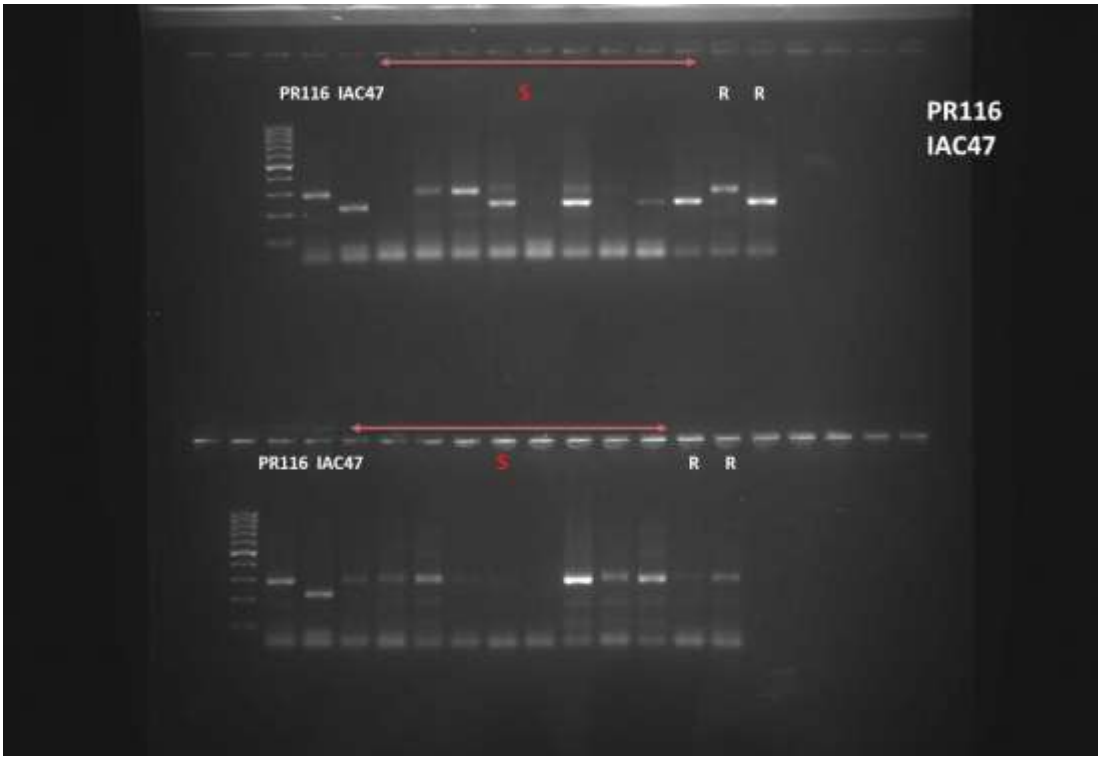


Plate 20: PCR based amplification of SSR marker RM25149 (chromosome 10) on parents PR116 and IAC47 as well as PR116 × IAC 47 mapping population. 'S' denotes susceptible plants while 'R' denotes resistant plants of mapping population.

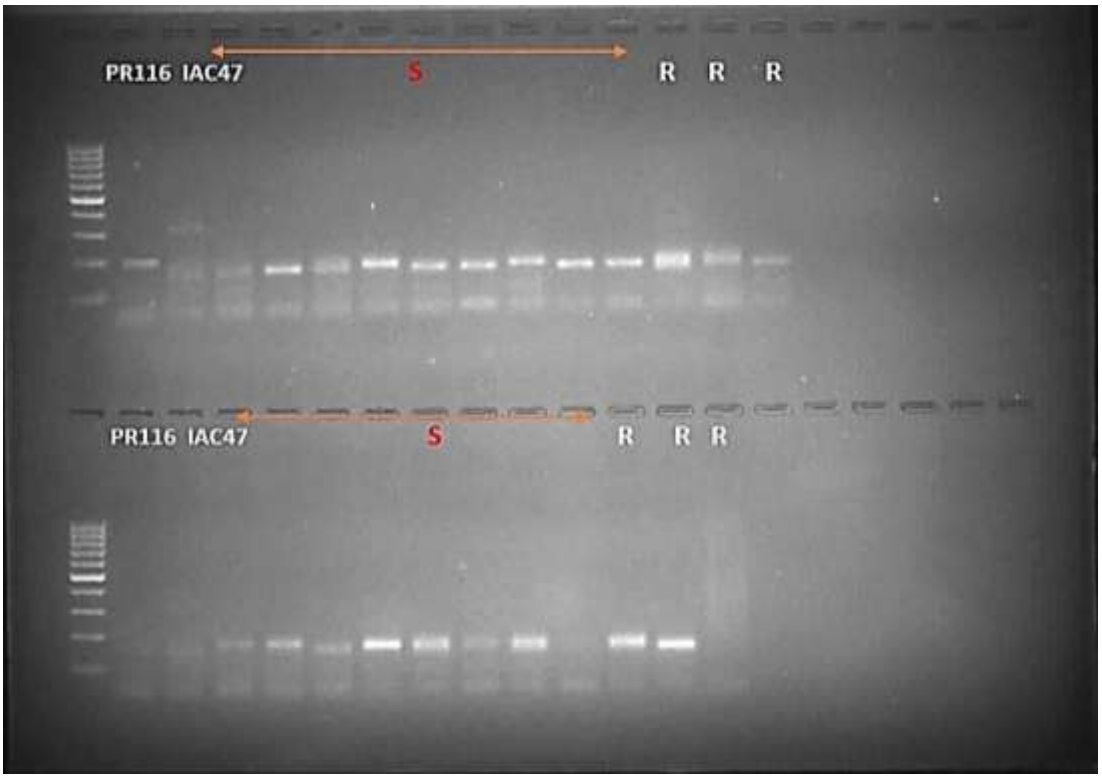


Plate 21: PCR based amplification of SSR marker RM10 (chromosome 5) on parents PR116 × IAC 47 and mapping population. 'S' denotes susceptible plants while 'R' denotes resistant plants of mapping population.

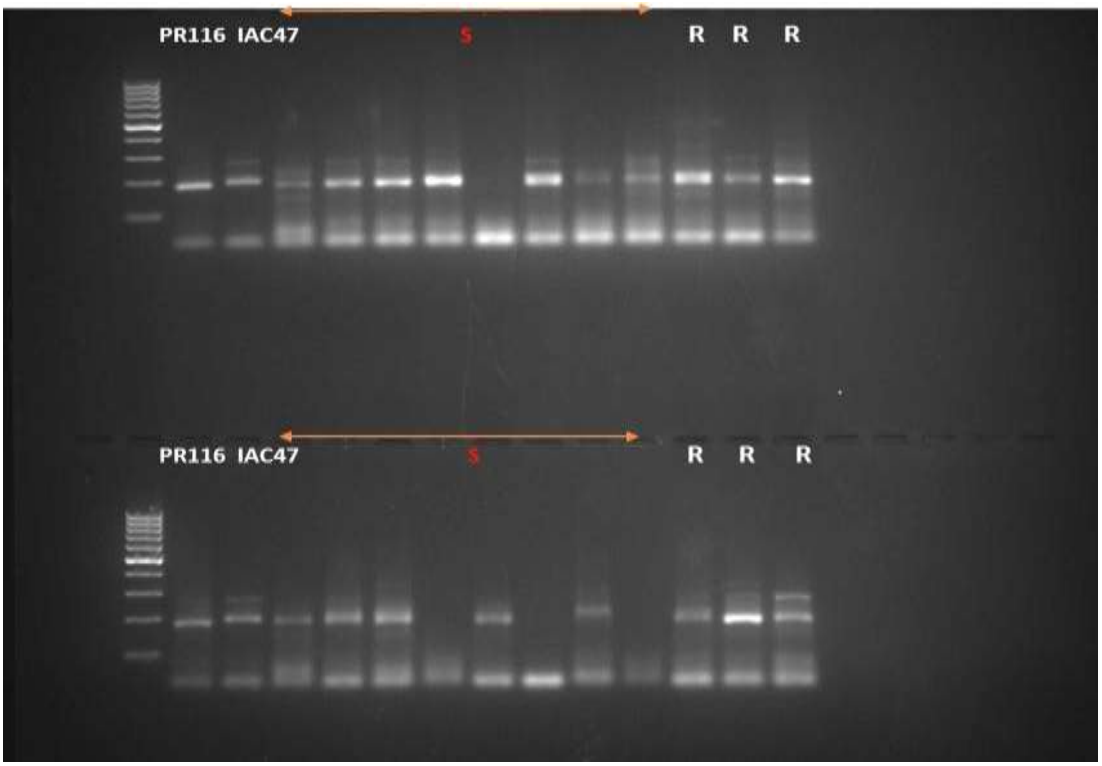


Plate 22: PCR based amplification of SSR marker RM18457 (chromosome 5) on parents PR116 × IAC 47 and mapping population. 'S' denotes susceptible plants while 'R' denotes resistant plants of mapping population.

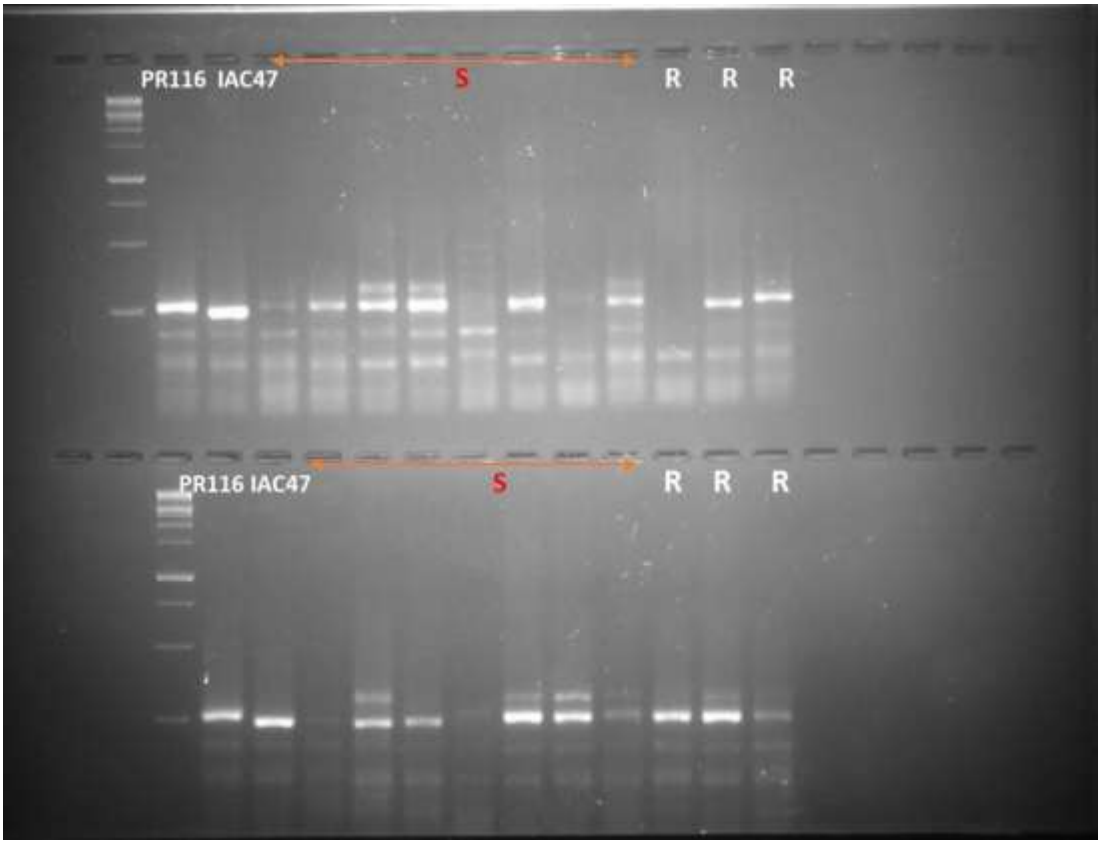


Plate 23: PCR based amplification of SSR marker RM28404 (chromosome 12) on parents PR116 × IAC 47 and mapping population. 'S' denotes susceptible plants while 'R' denotes resistant plants of mapping population.

polymorphic markers were used to identify variation in the mapping population. However, the identified polymorphic markers in this study fails to depict the association between the marker and false smut resistance due to lack of definite pattern of markers at loci.

Table 4.10: Genotypic data of SSR markers used on the mapping population

Chromosome	Marker	Polymorphism
1	RM3252	M
	RM3341	M
	RM3362	-
	RM104	P
	RM165	M
2	RM6307	M
	RM6139	M
	RM174	M
	RM6842	M
3	RM7642	M
	RM3654	-
	RM3372	M
	RM168	M
	RM85	-
4	RM7585	M
	RM16825	M
	RM17143	M
	RM16717	-
	RM6314	M
	RM16643	-
	RM17377	-
	RM349	M
	RM16493	P
5	RM6054	M
	RM7423	M
	RM7588	M
	RM289	P
	RM18457	P

Chromosome	Marker	Polymorphism
6	RM7583	-
	RM7434	P
	RM7158	-
	RM19623	M
	RM6818	-
	RM5814	M
7	RM6776	-
	RM6403	-
	RM3394	M
	RM3456	M
	RM21976	-
	RM248	-
8	RM8243	P
	RM23001	-
	RM6948	M
	RM22883	-
	RM22529	-
	RM264	P
9	RM23736	-
	RM5526	-
	RM3912	M
	RM3249	M
	RM1026	-
10	RM25149	P*
	RM216	-
	RM228	-
	RM239	-
	RM184	-
	RM474	M
11	RM26513	-
	RM27318	-
	RM27326	-

Chromosome	Marker	Polymorphism
11	RM27167	M
	RM6894	-
	RM10	P
	RM26643	P
	RM209	-
	RM286	-
	RM26063	-
12	RM6022	M
	RM27706	-
	RM101	-
	RM27962	M
	RM463	-
	RM6306	-
	RM465	M
	RM8404	P
	RM7315	M
	RM1159	-

Table 4.11: List of polymorphic markers identified in PR126 × CANAROXA F_2 mapping population

Chromosome	Marker
1	RM104
5	RM289
6	RM7434
8	RM8243
	RM264
10	RM25149
11	RM10
	RM26643

Table 4.12: List of polymorphic markers identified in PR116 × IAC 47 F_2 mapping population

Chromosome	Marker
4	RM16493
5	RM18457
10	RM25149
12	RM28404

Till date, 28 QTLs have been mapped using bi-parental populations from crosses of resistant and susceptible cultivars (Neelam *et al* 2021).

Using near isogenic introgression lines for disease resistance two QTLs were mapped (Xu *et al* 2002) using simple sequence repeat (SSR) markers. Li *et al* (2008) mapped two major genes controlling rice false smut with polygene mixed model. Li *et al* (2011) obtained seven QTL's controlling false smut resistance were on chromosomes 1, 2, 4, 8, 10, 11 and 12 respectively, from 157 recombinant inbred line population. Similarly, five QTLs were detected in Nanjing and Yangzhou respectively (Li *et al* 2014).

Ten QTLs that affect the incidence of disease were detected and used for mapping on rice chromosomes number 2, 3, 4, 6, 8, 10, 11 and 12 from 213 introgression lines obtained from a cross between Teqing (recipient) and Lemont (donor) using natural infection in China. (Zhou *et al* 2014). Han *et al* (2020) mapped five QTLs associated with resistance to false smut of rice on chromosomes 2, 4, 8, and 11 using 200 recombinant inbred lines in F_7 as mapping population using 179 polymorphic SSR markers. Recently, Qiu *et al* (2020) fine mapped single major locus, *FSR1* (*false smut resistance 1*) on chromosome 1 using an F_2 population derived from the cross between Nanjing11 (false smut resistant) and CG3 (false smut susceptible).

In GWAS analysis of rice false smut resistance, a total of 315 global diversity panel rice accessions were screened against *U. virens.* Three SNPs were found to be associated with three rice false smut resistance related traits *viz.*, disease plant percentage (DPP), disease panicles per disease plant (DPPDP) and disease smut balls per disease plant (DBPDP). (Long *et al* 2020). Hiremath et al (2021) screened 125 rice accessions from global rice diversity panel and GWAS for false smut related traits revealed significant associations on chromosome 2,3,6,9 and 11 for infected panicles per plant (IPP), number of smut balls per panicle (NSBP) on chromosomes 3 and 8, while for disease score (DS) on chromosomes 3, 4 and 11 of rice. Neelam *et al* (2021) mapped seven QTLs on rice chromosomes 2,4,5,7 and 9 using 2326 SNP markers on 250 recombinant inbred lines (RILs, F_9) derived from resistant breeding line RYT2668 (resistant) and Punjab Rice 116 (susceptible variety). QTL *qRFSr7.1b*, mapped on

chromosome 7 for the first time, was associated with four disease resistance proteins with NBS-LRR domain.

The present study on biology of the pathogen will be helpful in mitigating the risk management of false smut disease by development of suitable management strategies such as crop rotation and soil treatment with fungicides before sowing to check the soil-borne inoculum of *U. virens.* Realizing the importance of air-borne inoculum at the boot stage of crop, the use of fungicides especially copper-based fungicides is highly recommended before heading for the management of false smut under field conditions.

In 2002, PR116 covered 38% area in Punjab as major rice variety but it was highly susceptible to false smut. The disease causes widespread economic losses on this cultivar. Similarly, PR126 rice variety presently covered 14% area in Punjab. Both are high yielding rice varieties but highly susceptible to false smut which has hampered their cultivation in Punjab State. Till date no high level of resistance source has been found in rice germplasm. Breeding and utilization of resistant cultivar is the most effective and economical way to control false smut disease which ensure the high yield of crop. The above study aimed at screening of various rice accessions for identification of potential resistant sources to false smut of rice.

In the present study, we have screened rice germplasm lines for selection of putative donors resistant to false smut under field conditions. Out of 27 screened lines, we have selected two donors showing resistance to false smut in rice under field as well as artificial conditions. The two donors, CANA ROXA and IAC 47, were selected based on their minimum difference in days to flowering from the recipient parents selected, PR116 and PR126. The F_2 population of both the crosses (PR126 × CANAROXA and PR116 × IAC 47) were screened for resistance to false smut under field conditions. However, there was less incidence of false smut in both the population because of unfavourable weather conditions for development of disease at booting and late flowering stage of plants. We were expecting larger differences with respect to false smut trait in F_2 plants but there were only 23 susceptible plants from the population. The resistant plants were selected at random from both the population meanwhile not conformed about true to type resistance in plants. The less disease incidence in that year of screening poses difficulty in identification of true resistance in plants from the population. While the plants, which showed susceptible reaction under lower disease incidence, could be the plants showing highly susceptible reaction under otherwise high favourable weather conditions. DNA of selected susceptible and resistant plants along with parents were extracted and amplified with a set of SSR markers. The modified aim was to identify association of any marker with susceptible plants along with susceptible parents based on idea that any marker which is associated, will show similar band

size in all the susceptible plants and susceptible parents, while resistant parent will show polymorphism and random resistant plants may or may not show the same band of susceptible plants and susceptible parent. Further analysis of mapping population between the above prepared crosses (PR126 × CANAROXA and PR116 × IAC 47) will help to uncover the markers which are tightly linked to the false smut resistant QTLs. The 19 potential donors identified in the current study can be used in the various breeding programs for marker-assisted transfer of genetic resistance to these commercial and otherwise susceptible cultivars.

There were many drawbacks in finding the association of marker with false smut resistance trait during our present study. Firstly, we have screened the population under natural field conditions where the environmental conditions prevailing at that time were not highly conducive for the development of disease. The screening of rice accessions naturally under field conditions highly depends upon various environmental conditions for the incidence of this disease. Therefore, screening of large population under natural hotspots of false smut disease for 2-3 years will help us to phenotype plants precisely with false smut resistant trait.

Secondly, the screening of rice germplasm lines against this disease is done through artificial inoculation by boot injection method. This method requires certain controlled conditions for the disease development. We have inoculated plants under artificial conditions with boot injection method at boot stage of rice plants. This resulted in panicle sterility of plants and without disease. On the other hand, spray inoculation also could not produce desired results and there was no disease on plants except susceptible checks, which also shows less disease score.

Thirdly, based on the natural field conditions we have selected susceptible and resistant plants but with uncertainty of true to type resistance to false smut in selected resistant plants.

The differences in days to flowering should also be considered while selection of donors and recipient parents in preparation of crosses. This ensures synchronisation of most susceptible stage and further uniform distribution of disease in of both the parents. This helps in precise screening and identification of true-to-type resistant and susceptible plants in population.

The precise identification of disease and screening of plants against false smut of rice is very important for breeding to resistance against this disease. The disease is highly unpredictable under field conditions as it depends largely on the environmental conditions prevailing at the time of booting or late flowering stage of rice. The resistant plants can be selected from the population only if there is proper distribution of disease throughout the

population and categorization of plants among various disease reaction groups. This ensures selection of highly resistant and highly susceptible plants from the population and further genetic analysis. However, the natural hotspots of disease allow us to screen our large breeding population along with artificial inoculation of selected lines would help us to identify resistant sources of rice against the false smut.

CHAPTER V

SUMMARY

There are several diseases of rice and among these false smut is serious disease caused by an ascomycete fungus *Ustilaginoidea virens*. The perfect stage of the fungus includes *Villosciclava virens*. The disease is reported to cause 44% yield losses in Punjab on various rice varieties such as PR116 and PAU 201. The symptoms of the disease become visible only after flowering. The disease transform individual kernel in the panicle or few spikelets in the panicle into large, globose, velvety, yellowish spore mass in place of grains causing heavy direct losses to the rice growers. These smut balls are initially yellow in colour but later changes to orange and gradually to olive green. Keeping in view the seriousness of the disease, the present study entitled, "Biology of *Ustilaginoidea virens* (Cooke.) Takahashi and mapping QTL for resistance to false smut in rice (*Oryza sativa* L.)" was planned and conducted in the field experimental area, Department of Plant Breeding and Genetics and department of Plant Pathology, PAU, Ludhiana during the year 2017-21.

The pathogen was isolated axenically from the smut balls of the previous season crop on potato sucrose agar (PSA). The mass multiplication of the pure culture of *U. virens* was carried out in the flasks containing PSB incubated at 25±2°C for 8-10 days on shaker at 125 rpm. The DNA isolation of *U. virens* was formed from the mycelial mat formed in PSB after 10-15 days of constant shaking.

Out of the covered and uncovered panicles of the rice plant used for the source of air borne primary infection of the false smut pathogen, more incidence and per cent infected panicles were observed in the plants with uncovered panicle than covered panicle.

Of the infected and sterilized soil used for the source of primary infection of the disease, per cent infected panicles were noticed more in plants raised in the infected soil than in the sterilized soil.

Among the infected and healthy seed used for the source of primary infection of the pathogen, the per cent infected panicles were recorded only in the plants grown from infected seed. Of the six various treatments, the per cent infection of the panicle was recorded highest in the plants with uncovered panicles than the plants grown in infected soil. The plants raised from the infected seed shows only 5% infection. The pathogen does not survive in or on the seed and the seed do not serve as primary source of infection.

The nested PCR approach for asymptomatic detection of *U. virens* in rice tissues did not produce the desired results and require further standardization of primers for the detection of the pathogen from the rice tissues.

The field screening of 212 rice germplasm under 2K panel from IRRI was carried out based on 2015-17 screening disease score and 27 resistant lines with good agronomic traits

were selected. Among 27 naturally and artificially screened resistant lines, 19 lines were found completely free from the disease. These resistant lines can be further exploited in breeding programs for transferring resistance in otherwise susceptible commercial varieties of rice against the false smut disease.

Out of the 19 selected resistant lines, two lines CANAROXA and IAC 47 were used as donors for making crosses between PR126 × CANAROXA and PR116 × IAC 47 respectively. Most of the susceptible F_2 plants of the cross between PR126 × CANAROXA exhibited disease score 5 based on the percentage of infected panicles while maximum score was 7 in three plants. The same trend was observed in population of PR116 × IAC 47.

Out of the 78 SSR primer pairs, marker RM25149 were found to be polymorphic in parents of both the crosses (PR126 × CANAROXA and PR116 × IAC 47). The markers which show polymorphism in PR116 × IAC 47 includes RM16493, RM18457, RM25149 and RM28404 on chromosome 4, 5, 10 and 12, respectively. On the other hand, the polymorphic markers in case of PR126 × CANAROXA includes RM104, RM289, RM7434, RM8243, RM264, RM25149, RM10 and RM26643 on chromosome 1, 5, 6, 8, 10 and 11, respectively. The identified polymorphic markers failed to depict the association between the marker and false smut resistance gene due to lack of definite pattern of markers at loci. Further analysis in next generation will be helpful to uncover the association between the marker and the false smut resistance gene.

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