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RESEARCH ARTICLE

Standardizing Protocol for Developing Pure Inoculum of Leaf Crinkle Pathogen in Blackgram [*Vigna Mungo* (L.) Hepper] For Further Characterization

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ABSTRACT

Blackgram [*Vigna mungo* (L.) Hepper] or urdbean is one of the most important leguminous crops cultivated in India, Pakistan and South-East Asian countries. The crop is known to be affected by various biotic and abiotic stresses. Among the biotic factors, Urdbean Leaf Crinkle Disease (ULCD) is an economically significant one that has devastated crops in major blackgram cultivating regions. ULCD causes severe crinkling, rugosity of lamina, stunting of plants and malformation of buds inflicting heavy yield losses. Nevertheless, the disease was reported many decades ago and resulted in economic damage to the crop throughout all the seasons; the disease was reported many decades ago and resulted in economic damage to the crop throughout all the seasons; the causal agent of the disease is still unknown. In order to find the etiology and characterize at the molecular level, state-of-the-art technology, viz., Next Generation Sequencing / High Throughput Sequencing, has to be employed. Due to the occurrence of ULCD at field level as mixed infection along with other major viruses infecting blackgram like yellow mosaic, leaf curl, stem necrosis etc., the maintenance of pure inoculum under glasshouse conditions is an essential prerequisite. In the present study, protocol for modified seed sprout abrasion method was explained that helps in maintenance of pure inoculum of ULCD infected blackgram plants under glasshouse conditions in which the symptoms started appearing 20 days post inoculation (DPI) and the disease incidence was up to 88%. Further investigation has proved this modified sprout seed abrasion method as efficient protocol to obtain transmission at higher levels at the earliest.

Keywords: Sap Inoculation, Modified Sprout Seed Abrasion, Blackgram, Leaf Crinkle, Pure Inoculum.

INTRODUCTION

Blackgram [*Vigna mungo* (L). Hepper] or Urdbean is one of the most predominantly cultivated crops of '*Vigna*' group belonging to 'Leguminosae' family. India is the primary origin of blackgram whereas, Central Asia as secondary center (Vavilov, 1926) and is cultivated from ancient times. This food legume is highly-priced, a good source of protein, minerals, and energy, and is used in daily diaries. The major urdbean-producing states in India are Madhya Pradesh, Uttar Pradesh, Punjab, Maharashtra, West Bengal, Tamil Nadu, Rajasthan, Andhra Pradesh and Karnataka computing for 91 per cent of total urdbean cultivation of the country with the production of 2.84 million tonnes from 4.76 Mha area in India which is accounting for 70% of the global production (Agricultural Statistics, DES, MoAF&W, 2021-22).

Though black gram contributes 11% of total pulse production in India, over the years, production and productivity of black gram have remained stagnant due to the biotic stresses, and virus diseases, in particular, are the most important contributors to low production and productivity (Biswas *et al.,* 2009) in India. Among

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the diseases, Urdbean Leaf Crinkle Disease (ULCD) is causing heavy yield loss up to 64% (Latha *et al.,* 2022). The disease was first reported in India by Nariani (1960) from Delhi and in Tamil Nadu it was reported by Narayanasamy and Jaganathan (1973). The symptoms are characterized by the appearance of extreme crinkling, curling, puckering, rugosity of leaves, reduction in internodal length as a result stunting of plants and malformation of floral organs (Williams *et al.,* 1968; Nene, 1968; Bindra, 1971; Khatri *et al.,* 1971; Subbarao, 1984; Kolte and Nene 1972; Reddy *et al.,* 2005; Brar and Rataul, 1987). The causal agent is still classified as uncharacterized due to ambiguity in identifying the etiology and determining the vector association, in transmission.

Although the causative agent of the disease is considered to be a virus by many researchers, the exact genus, species, type of genome, morphology of the virus particle, and mode of transmission have not yet been confirmed. The Next Generation sequencing (NGS) or deep sequencing (DS) or high throughput sequencing (HTS) is a recent advancement and powerful substitute for the diagnosis, detection, and characterization of the total number of pathogens, including viruses in symptomatic or asymptomatic samples.

Efforts of the earlier research workers (Williams *et al*., 1968; Chohan and Kalia, 1967) failed to transmit ULCD through sap inoculation. Later, Kolte and Nene (1972) succeeded in the mechanical transmission of the causal virus using potassium phosphate buffer (PPB) (pH 7.6) as an extracting medium and carborundum as an abrasive. The incubation period ranged from 11-16 days in the plant for symptom expression. As various authors reported differently regarding the efficacy of ULCD inoculation methods *viz.,* sap inoculation on to 2 leaf stage and sprout seed abrasion method, the present study was undertaken with an aim to demonstrate the most effective and reliable method of modified sprout seed abrasion method for the development and maintenance of pure inoculum of ULCD infection in blackgram.

MATERIAL AND METHODS

The experiments were conducted in the glasshouse of Department of Plant Pathology of Tamil Nadu Agricultural University (TNAU), Coimbatore.

Source of initial inoculum

In order to develop the initial inoculum, black gram plants showing typical symptoms of ULCD at experimental fields of Tamil Nadu Agricultural University, Coimbatore, was tagged at the initial stages of ULCD symptomatic plants, and the seeds were collected at the time of harvest. Those seeds were sown in pots and kept under insect proof cages of glasshouse. Leaves showing typical leaf crinkle symptoms were collected from glasshouse sown plants and used as initial inoculum for further studies.

Inoculation methods

For the maintenance of pure inoculum under glasshouse conditions, both mechanical sap inoculation as well as sprout seed abrasion methods of inoculation as described by Biswas *et al.* (2012) were followed. Comparing the two methods, the highly effective sprout seed abrasion method was modified with pin pricking and addition of combinations of abrasives and additives.

Mechanical sap inoculation

For mechanical transmission, ULCD infected symptomatic leaves were collected freshly and macerated with 0.05 M PPB (pH 7.0) using 0.1% of 2-mercaptoethanol (2-ME) in a sterilized and pre-chilled pestle and mortar. Before inoculation, carborundum powder was dusted over the leaf lamina as an abrasive. The homogenized crude suspension was rubbed gently onto Blackgram cv. VBN 8 at two leaf stage. Inoculated seedlings were gently washed with distilled water using a squeeze bottle to remove the excess inoculum. Plants, along with separate buffer control, were kept under insect-proof cages in a glasshouse, and observations were recorded up to 55 days post-inoculation (DPI).

Sprout seed abrasion method

Seeds of blackgram cv. VBN 8 was soaked in distilled water for 6 hrs. Pre-soaked seeds were placed on moist blotter paper for 8 hrs for the sprouts to emerge (Biswas *et al.,* 2012). One gram of infected leaf sample was macerated in 5 mL of PPB buffer (0.05M, pH 7.0) [(0.05M 2.335g of Dipotassium hydrogen phosphate in 500mL of sterile distilled water was used as Sol A, while 1.575 g of Potassium dihydrogen orthophosphate in 500mL of sterile distilled water was used as Sol B. To get the desired combination and pH, 38.9mL of Sol A and 61.1mL of Sol B are combined] supplemented with 0.1% 2-ME in a sterilised and chilled ice-cold mortar and pestle. Seeds were soaked in crude sap suspension along with carborundum as

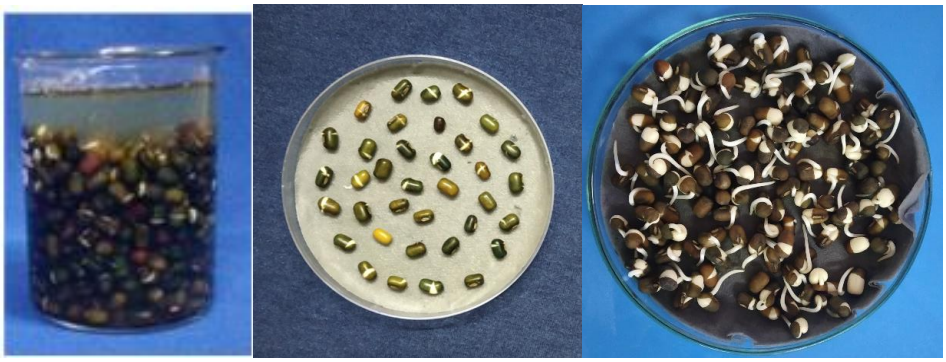
abrasive and incubated for one hour with intermittent shaking (Fig 1.). and sown in pots (dia. 20") given with nutrient supplement, timely irrigation, and kept under insect-proof cages. Sprouted seeds soaked in buffer was used as control.

Modified sprout seed abrasive method

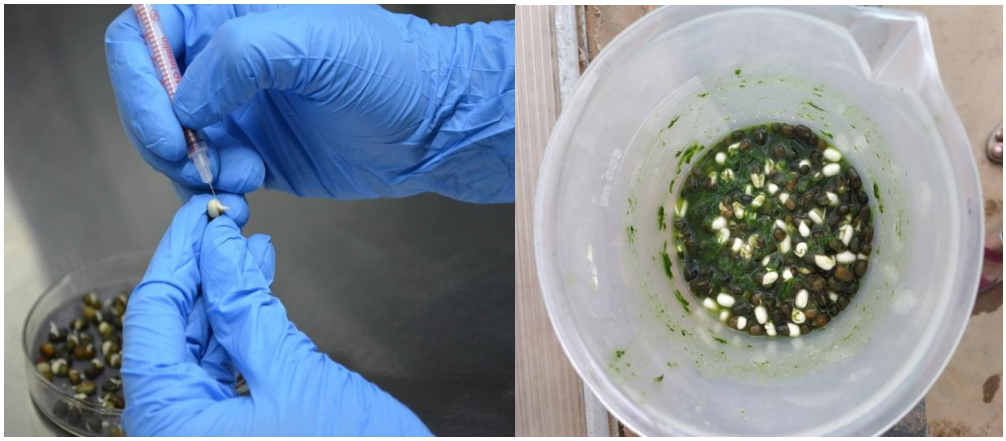
The protocol was standardized by modifying the method described by Biswas *et al*. (2012). Abrasives and additives were added to obtain more symptom transmission and to get the infection ULCD in blackgram plants at the earliest. The present study was conducted using blackgram (cv. VBN 8). Two sets of sprouted seeds were incubated in crude sap and were followed (i) with pin pricking and (ii) without pin pricking. The crude suspension was ground with 0.05 M PPB (pH 7.0) in 1:5 dilution with different combinations of additives and abrasives. In pin prick method of sap inoculation, 2-3 gentle pricks were made to the emerging meristem of sprouted seeds by wounding with a needle (BD U-40 Ultra fine 6mm syringe) before soaking in the ULCD infected leaf sap. In the method without pin pricking, the sprouted seeds were, as such, soaked in the ULCD-infected leaf sap.

To evaluate the effect of antioxidants, four different combinations of inoculum *viz.*, 0.05M PPB (pH 7.0) alone, PPB (pH 7.0) containing 0.15 % Sodium Sulphite (SS), PPB containing 0.1% 2-ME and PPB with 0.15 % SS + 0.1% 2-ME were prepared. Similarly, to determine the role of abrasives on percent transmission of disease, the sap was divided into four parts, i.e., without abrasives, with 2% carborundum (320 grit), with 1% elite (545) and both 2 % carborundum + 1 % Celite. Seeds soaked in crude sap of infected leaves were sown in pots (20 x 20 cm) filled with potting mixture consisting of red soil, farmyard manure, vermiculite, and sand in the ratio of 3:2:2:1 (W/W). Thirty plants for each parameter (6 pots: 5 plants/ pot) in three replicates were used for mechanical transmission under insect-proof conditions along with respective buffer control. The disease incidence was recorded from 14 to 55 DPI.

Fig 1. Optimisation of Sprout Seed Abrasion Method



(a) Soaking the seeds **(b) Sprouting the seeds on moist blotter paper**



(c) Pin pricking on the sprouted seeds **(d) Soaking the pin pricked seeds inULCD infected leaf sap**

RESULTS AND DISCUSSION

Seeds from symptomatic ULCD-affected plants were tagged, collected at the time of harvest, and sown in insect-proof conditions for ULCD inoculum maintenance. Symptoms observed from these plants were similar to that of field *viz.,* crinkling of the lamina, enlargement of leaf, and malformation of buds, and these symptomatic leaves were further used for sap inoculation.

The crude sap was prepared from infected leaf samples maintained in the glasshouse and inoculated onto healthy black gram plants cv. VBN 8 on 7th day (2 leaf stage). Periodic observations were recorded starting from 14 DPI up to 55 DPI. Mild crinkling symptoms were first observed on 3rd trifoliate stage (25 DPI). The symptom progressed as lamina crinkled and puckered, and no flowering was observed up to 55 DPI, whereas enlargement of leaf lamina was more prominent. Disease incidence with sap transmission on two leaf stage was 84% (Table1). No symptoms were observed in un-inoculated control plants. The results confirmed that the ULCD is sap transmissible when inoculated on to healthy plants at primary leaf stage.

The sprouted seeds incubated in infected leaf sap were sown in pots and maintained under insect-proof greenhouse conditions. Observations were taken based on ULCD symptoms, and the percent disease incidence was recorded (88%) (Table 1). Sprout seed abrasion method showed a higher percent disease incidence compared to sap inoculation on to two leaf stage. Hence, this method was further carried for standardization of protocol for efficiency of transmission and earliest onset of symptoms under glasshouse conditions.

There were significant differences in per cent disease transmission with different treatment combinations of buffer, abrasives, additives and inoculation methods applied (Table 2a, 2b). Among the inoculation procedures tested, maximum transmission efficiency was obtained with pin pricking method, when infected leaf suspension was prepared with PPB supplemented with SS + 2-ME and celite and carborundum as abrasive, while symptom expression also started at the earliest at 2nd trifoliate stage and reached maximum (83 - 100%) at 20 DPI followed by PPB supplemented only with 2-ME (60-100%) expressing symptoms at 2nd trifoliate stage.

Comparing various combinations of additives employed with the buffer, PPB + SS + 2-ME showed 40-95% transmissibility followed by SS alone (21-92%) at 25th DPI, while 2-ME alone resulted in the transmission of 21-92% at 30th DPI. Symptoms developed were very similar to the field symptoms with the above method (Fig 2e&f). Symptoms started with the crinkling of the lamina (Fig 2a) followed by puckering, downward curling (Fig 2b), and enlargement of leaves (Fig 2c). Malformation of floral buds (Fig 2d) and stunted growth was also observed (Fig 2 d).

By inoculating 6-day old seedlings with ULCV, Kolte and Nene (1972) achieved 100% transmission. Rao and Reddy (2005) found that artificially inoculated ULCV in blackgram plants at primary leaf stage produced initial symptoms 10 to 12 days after inoculation and highest susceptibility was observed at primary leaf stage.

In a study to increase the effectiveness of mechanical transmission in watermelon genotypes for *Watermelon bud necrosis orthotospovirus,* a protocol was standardized by studying the effect of different

Table 1. Effect of inoculation methods on per cent disease incidence of ULCD in blackgram

Method of Inoculation	Total no. of plants inoculated	Number of plants showing symptoms days after post inoculation (DPI)									Days required for initial symptom development (DPI)	Mean incidence (%)	Healthy control
		14 DPI	20 DPI	25 DPI	30 DPI	35 DPI	40 DPI	45 DPI	50 DPI	55 DPI			
Sap inoculation at 2-Leaf stage	50	0	0	9	15	21	28	32	42	42	28 days	84.0	0
Sprout seed abrasion	50	0	11	13	21	32	37	44	44	44	25 days	88.0	0

Table 2a: Per cent transmission of leaf crinkle disease in blackgram (cv. VBN 8) using various Inoculation method, Abrasive and Additives

Inoculation Methods	Abrasives	Type of buffer combination	Transmission % at different day post inoculation (DPI) stages based on symptoms									Days required (DPI)	Stage of the crop at which symptoms expressed
			14	20	25	30	35	40	45	50	55		
Without Pin Prick	No abrasive	PPB (pH 7.0)	0	0	0	0	8	10	10	10	10	35	4th trifoliate
		PPB + 2-ME	0	0	0	0	9	10	13	13	13	35	4th trifoliate
		PPB + SS	0	0	0	0	9	10	13	15	15	35	4th trifoliate
		PPB + SS + 2-ME	0	0	0	9	10	10	10	16	16	30	4th trifoliate
	Celite	PPB (pH 7.0)	0	0	0	0	9	10	10	10	10	35	4th trifoliate
		PPB + 2-ME	0	0	0	14	14	15	20	20	20	30	4th trifoliate
		PPB + SS	0	0	0	15	15	15	20	20	20	30	4th trifoliate
		PPB + SS + 2-ME	0	0	0	16	19	19	19	24	24	30	3rd trifoliate
	Carborundum	PPB (pH 7.0)	0	0	0	0	15	21	32	32	32	35	4th trifoliate
		PPB + 2-ME	0	0	0	21	32	32	43	43	43	30	4th trifoliate
		PPB + SS	0	0	0	19	23	33	42	42	42	35	4th trifoliate
		PPB + SS + 2-ME	0	0	0	35	46	53	53	53	53	25	3rd trifoliate
	Celite + Carborundum	PPB (pH 7.0)	0	0	0	0	15	15	25	25	25	35	4th trifoliate
		PPB + 2-ME	0	0	0	15	21	21	33	33	33	30	4th trifoliate
		PPB + SS	0	0	0	21	32	32	42	42	42	30	4th trifoliate
		PPB + SS + 2-ME	0	0	40	40	46	53	53	53	53	25	3rd trifoliate
		Sem	0	0	2.74	3.14	3.16	3.18	3.29	3.30	3.40		
		CD	0	0	7.69	8.83	8.88	8.93	9.25	9.25	9.55		

*Significant differences were obtained at P = 0.05; PPB: Potassium phosphate buffer; SS: Sodium Sulfite; 2-ME: Mercaptoethanol.

Table 2b: Per cent transmission of leaf crinkle disease in blackgram (cv. VBN 8) using various Inoculation method, Abrasive and Additives

Inoculation Methods	Abrasives	Type of buffer combination	Transmission % at different day post inoculation (DPI) stages based on symptoms									Days required (DPI)	Stage of the crop at which symptoms expressed
			14	20	25	30	35	40	45	50	55		
Pin prick method	No abrasive	PPB (pH 7.0)	0	0	0	0	15	15	16	16	16	35	4th trifoliate
		PPB + 2-ME	0	0	0	19	21	21	33	46	46	30	4th trifoliate
		PPB + SS	0	0	0	15	21	32	42	42	42	30	4th trifoliate
		PPB + SS + 2-ME	0	0	0	32	36	42	46	53	53	30	3rd trifoliate
	Celite	PPB (pH 7.0)	0	0	0	9	15	21	32	32	32	30	3rd trifoliate
		PPB + 2-ME	0	0	10	21	21	42	53	53	62	25	3rd trifoliate
		PPB + SS	0	0	21	32	53	72	82	82	82	25	3rd trifoliate
		PPB + SS + 2-ME	0	0	40	53	53	85	85	85	85	25	3rd trifoliate
	Carborundum	PPB (pH 7.0)	0	0	0	21	32	42	42	56	56	30	4th trifoliate
		PPB + 2-ME	0	0	21	42	53	64	72	72	85	25	3rd trifoliate
		PPB + SS	0	0	53	53	72	76	85	85	85	25	3rd trifoliate
		PPB + SS + 2-ME	0	0	91	94	95	100	100	100	100	25	3rd trifoliate
	Celite + Carborundum	PPB (pH 7.0)	0	0	83	83	92	92	92	94	94	30	4th trifoliate
		PPB + 2-ME	0	60	92	92	93	93	94	95	100	20	2nd trifoliate
		PPB + SS	0	0	92	92	92	94	95	95	100	25	3rd trifoliate
		PPB + SS + 2-ME	0	83	95	95	100	100	100	100	100	20	2nd trifoliate
		Uninoculated control	0	0	0	0	0	0	0	0	0	-	-
		Sem	0	2.86	3.18	3.39	3.23	3.41	3.56	3.61	3.98		
		CD	0	8.04	8.92	9.52	9.08	9.58		10.13			

*Significant differences were obtained at P = 0.05; PPB: Potassium phosphate buffer; SS: Sodium Sulfite; 2-ME: Mercaptoethanol.

Fig 2. Symptoms developed by standardized sprout seed abrasive method

(a) Crinkling of lamina (b) Puckering and downward curling

(c) Enlargement of lamina Malformation of floral bud

(e) Inoculated plants through modified sprout seed abrasion method with pin prick
(f) Buffer inoculated plants

inoculation methods and buffer formulations on virus transmission and validated in 17 watermelon genotypes (Jain *et al.,* 2018). Sreevathsa *et al.* (2012) developed efficient sap inoculation technique method for *Tobacco streak virus* (TSV) by injuring 2-day old seedling at the meristem and immersing in the serially diluted sap and attained 80% transmission at 10^{-5} dilution. Pensuk *et al.* (2002) adopted mechanical inoculation to screen peanut genotypes for resistance to Peanut bud necrosis virus (PBNV) in both field

and glasshouse environments and reported 60-100 percent transmissibility in susceptible genotypes and consequently, in 2010 he studied the relationship of temperature and relative humidity on the efficacy of PBNV mechanical inoculation on peanuts. From the earlier studies, it was evidenced that the optimization of mechanical transmission protocol will improve the transmission efficiency of the virus. The authenticity and reproducibility of the methodology were further confirmed, and it can be used for the maintenance of pure inoculum as well as for artificially screening the genotypes against ULCD both under field and glasshouse conditions.

CONCLUSIONS

In the present study seeds were mechanically sap inoculated by sprout seed abrasion method as well as on to 2-leaf stage. The influence of artificial inoculation on ULCD incidence has been studied on the VBN 8 cultivar. The present investigation is helpful to suggest that the ULCD is transmitted by mechanical inoculation through both the methods whereas, leaf crinkle infection is picked up by host plants before germination and symptom expression is early, suggesting the modified sprout seed abrasion approach is effective. As new viruses causing mixed infections in pulse crops emerge, keeping the virus inoculum free of vector-borne contamination is essential for determining the virus diagnosis, host specificity and symptom expression. Since the causative agent of ULCD is still unknown, maintaining a pure inoculum will help eliminate mixed infections and investigate host pathogen interactions.

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