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Optimization of Sprout Seed Abrasion Method for Efficient Inoculation of Leaf Crinkle Disease in Urdbean [*Vigna mungo* (L.) Hepper]

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ABSTRACT

Background: Urdbean leaf crinkle is one of the serious diseases of urdbean (*Vigna mungo* (L.) Hepper) which is caused by an ungrouped virus called Urdbean leaf crinkle virus (ULCV). The disease is highly seed transmissible under natural conditions and in experimental conditions it is mechanically transmitted through sap inoculation.

Methods: The aim of this study was to standardize the protocol for sap inoculation by sprout seed abrasive method by comparing the effects of different inoculation methods, such as pin prick and without pin prick, with 0.05M potassium phosphate buffer (pH 7.0) supplemented with additives and abrasives and to further validate the standardized protocol upon different urdbean genotypes.

Result: This is the first effort on the optimization of protocol in which, pin pricking as inoculation method with potassium phosphate buffer (0.05M, pH 7.0) supplemented with 0.15% sodium sulfite+0.01M 2-Mercaptoethanol and 1% celite+2% carborundum showed 100% transmissibility foremost at 2nd trifoliate stage and further validation with various urdbean genotype resulted in percent transmission ranging from 92 to 100% which evinces the efficacy and repeatability of the methodology.

Key words: Additives, Inoculum, Leaf Crinkle disease, Optimization of protocol, Sprout seed abrasion.

INTRODUCTION

Urdbean (*Vigna mungo* (L.) Hepper) or Blackgram, mungo bean or black *matpe* bean is one of the important pulse crops cultivated in the South Asia, widely adopted to both subtropical and semi-arid areas (Ganguly and Bhat, 2012). The area under cultivation of urdbean in Tamil Nadu during *Kharif* 2019-20 is 0.36 lakh ha with 0.33 lakh tonnes production (Department of Economics and Statistics, 3rd Advance estimates 2019-20). Nonetheless, India has largest acreage and is the highest consumer of urdbean, however production is constrained due to prevalence of biotic stresses.

Among the biotic stresses, Urdbean leaf crinkle disease (ULCD) is a destructive viral disease that infects urdbean and has become a potential danger to the crop in recent years since most of the cultivars are susceptible to the disease (Gautam *et al.* 2016). Symptom expression is highly influenced by temperature (Baranwal *et al*., 2019; Binyamin *et al.,* 2011) and causes high yield reductions (Baranwal *et al*., 2021; Priyanga, 2021; Sravika, 2018; Sharma *et al.,* 2015; Kanimozhi *et al.,* 2009; Mandhare, 2007). Mechanical inoculation of Leaf crinkle disease onto different cultivars by sprout seed abrasive method was followed by Biswas *et al. (*2012). Dubey *et al*. (2020) conducted mechanical inoculation of leaf crinkle disease onto different stages of urdbean plant and observed 100% disease incidence when inoculated onto germinated seeds. In accordance with various investigations on inoculation of leaf crinkle disease carried by various workers, the present work was outlined to standardize the sprout seed abrasion method in order to increase the transmission efficiency. In an attempt to identify viruses associated with leaf crinkle disease in mungbean and urdbean by Next Generation Sequencing (NGS) of small RNAs from field samples, presence of Cowpea mild mottle virus (CpMMV) was detected but its exact role in causing leaf crinkle symptom and host-pathogen interaction was not established (Baranwal *et al*., 2015).

In recent years, a variety of methods have been employed to study novel plant virus which include Next generation deep sequencing of infected samples followed by bioinformatics analysis. In the process of identifying the etiology of uncharacterized disease through NGS, some of the asymptomatic viruses present in the field sample are always interfering which suggests the need for the maintenance of pure inoculum under glasshouse conditions. These viral inoculation investigations are influenced by a variety of factors such as plant age, inoculum source, buffer

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with antioxidants and abrasives, anti-inhibitors of sap transmission and so on. As a result, most attention has been placed on the standardization of effective high throughput sap inoculation procedures for viruses (Mandal *et al.,* 2002, 2008; Laidlaw, 1987). In order to identify the putative virus associated with ULCV in urdbean by NGS, it is essential to maintain the virus inoculum under insect proof conditions. Therefore, present investigation was carried out to optimize the inoculation procedure by sprout seed abrasive method and its validation with different genotypes.

MATERIALS AND METHODS

Collection and maintenance of the infected plants

As the leaf crinkle disease is highly seed transmissible, symptomatic plants in the experimental farms at Tamil Nadu Agricultural University were identified, tagged during summer 2020 and seeds from those infected plants were collected at the time of harvest and were sown in the glasshouse during *kharif* 2020. Symptomatic leaves developed were used as inoculum for further use.

Optimization of mechanical inoculation protocol

The present protocol was optimized by modifying the method described by Biswas *et al*. (2012) in the inoculation procedure by adding abrasives and additives to obtain high symptomatic transmissibility and to attain the infection on urdbean plants at the earliest. The present study was performed during *rabi* 2020-21 using VBN 8 cultivar of urdbean where in, two methods of incubating sprouted seeds in the leaf crinkle infected leaf sap were followed (i) with pin prick of the sprouted seed and (ii) without pin pricking, where in the virus suspension was grinded with 0.05 M PPB (pH 7.0) with different combinations of additives and abrasives. In pin prick method of sap inoculation, 2-3 gentle pricks were made to the emerging meristem by wounding with a fine needle (Fig 1A).

To evaluate the effect of antioxidants, four different combinations of inoculum was prepared *viz*., 0.05M PPB (pH 7.0) containing 0.2% Sodium Sulphite (SS), PPB containing 0.01M 2-Mercaptoethanol (2-ME) and PPB with 0.15% SS + 0.01M 2-ME. Similarly, to determine the role of abrasives on percent transmission of virus, the sap was divided into three parts *i.e.,* only 2% carborundum (320 grit), only 1% celite (545) and both 2% carborundum + 1% celite. Seeds soaked in infected leaf suspension were sown in pots (20 x 20 cm) filled with potting mixture consisting of red soil, farm yard manure, vermiculite and sand in the ratio of 3:2:2:1 (W/W). Total 30 plants for each parameter (6 pots: 5 plants/pot) for three replications along with respective healthy control were maintained under 28±2°C and percent transmission was recorded from 14 to 55 Days post inoculation (DPI).

Validation of the optimized protocol

Following the optimization of the mechanical inoculation to urdbean by sprout seed abrasion method, the experiment was extended for protocol validation. Nine cultivated urdbean genotypes *viz.,* VBN4, VBN5, VBN6, VBN9, VBN10, VBN11, MDU-1, CO 6 including the susceptible check variety CO5 were obtained from National Pulse Research Centre, Vamban, Tamil Nadu and Department of Pulses, Tamil Nadu Agricultural University (TNAU). All the sprouted seeds of different genotypes were mechanically inoculated with standardised method and buffer control (without any inoculation) was maintained for all the genotypes separately in the glasshouse.

Statistical analysis

Analysis of variance was performed on the data (ANOVA). For analysis, the per cent data was arcsine transformed. A completely randomised design was used to calculate the standard error of mean (SEm), standard error of deviations (SEd) and critical difference (C.D.) values (Gomez and Gomez, 1984).

RESULTS AND DISCUSSION

Effect of inoculation methods

Among the inoculation procedures tested, maximum transmission efficiency (83-100%) 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 (Table 1), while symptom expression started at the earliest at 2nd trifoliate stage and reached maximum (100%) at 20 DPI followed by PPB supplemented with only 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 at 25th DPI followed by SS alone and 2-ME alone (21-92%) at 30th DPI, which indicate that both SS and 2-ME show same efficacy when supplemented alone with the buffer. Similarly, while comparing the effect of abrasives used carborundum + celite showed a maximum of 40-95% transmissibility at 25th DPI followed by carborundum (35-92%) and celite (16-53%) alone at 30 DPI which shows that carborundum when used alone is more effective than celite. There was a significant difference between the buffer, abrasives, additives and inoculation method and minor variations were observed in the severity of symptoms across various treatments.

In a study to increase the effectiveness of mechanical transmission in watermelon genotypes for *Watermelon bud necrosis orthotospovirus,* protocol was standardized by studying the effect of different 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 per cent transmissibility in susceptible genotypes and consequently, in 2010 he studied the relationship of temperature and relative humidity on the

Table 1: Percent transmission of leaf crinkle disease in urdbean (cv. VBN 8) using various inoculation methods, abrasives 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	Healthy control
			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	4^{th} trifoliate	-
		PPB+2-ME	0	0	0	0	9	10	13	13	13	35	4^{th} trifoliate	-
		PPB+SS	0	0	0	0	9	10	13	15	15	35	4^{th} trifoliate	-
		PPB+SS+2-ME	0	0	0	9	10	10	10	16	16	30	4^{th} trifoliate	-
	Celite	PPB (pH 7.0)	0	0	0	0	9	10	10	10	10	35	4^{th} trifoliate	-
		PPB+2-ME	0	0	0	14	14	15	20	20	20	30	4^{th} trifoliate	-
		PPB+SS	0	0	0	15	15	15	20	20	20	30	4^{th} trifoliate	-
		PPB+SS+2-ME	0	0	0	16	19	19	19	24	24	30	3^{rd} trifoliate	-
	Carborundum	PPB (pH 7.0)	0	0	0	0	15	21	32	32	32	35	4^{th} trifoliate	-
		PPB+2-ME	0	0	0	21	32	32	43	43	43	30	4^{th} trifoliate	-
		PPB+SS	0	0	0	19	23	33	42	42	42	35	4^{th} trifoliate	-
		PPB+SS+2-ME	0	0	0	35	46	53	53	53	53	30	3^{rd} trifoliate	-
	Celite+ Carborundum	PPB (pH 7.0)	0	0	0	0	15	15	25	25	25	35	4^{th} trifoliate	-
		PPB+2-ME	0	0	0	15	21	21	33	33	33	30	4^{th} trifoliate	-
		PPB+SS	0	0	0	21	32	32	42	42	42	30	4^{th} trifoliate	-
		PPB+SS+2-ME	0	0	40	40	46	53	53	53	53	25	3^{rd} trifoliate	-
Pin prick method	No abrasive	PPB (pH 7.0)	0	0	0	0	15	15	16	16	16	35	4^{th} trifoliate	-
		PPB+2-ME	0	0	0	19	21	21	33	46	46	30	4^{th} trifoliate	-
		PPB+SS	0	0	0	15	21	32	42	42	42	30	4^{th} trifoliate	-
		PPB+SS+2-ME	0	0	0	32	36	42	46	53	53	30	3^{rd} trifoliate	-
	Celite	PPB (pH 7.0)	0	0	0	9	15	21	32	32	32	30	3^{rd} trifoliate	-
		PPB+2-ME	0	0	10	21	21	42	53	53	62	25	3^{rd} trifoliate	-
		PPB+SS	0	0	21	32	53	72	82	82	82	25	3^{rd} trifoliate	-
		PPB+SS+2-ME	0	0	32	53	53	85	85	85	85	25	3^{rd} trifoliate	-
	Carborundum	PPB (pH 7.0)	0	0	0	21	32	42	42	56	56	30	4^{th} trifoliate	-
		PPB+2-ME	0	0	21	42	53	64	72	72	85	25	3^{rd} trifoliate	-
		PPB+SS	0	0	53	53	72	76	85	85	85	25	3^{rd} trifoliate	-
		PPB+SS+2-ME	0	0	91	94	95	100	100	100	100	25	3^{rd} trifoliate	-
	Celite+ Carborundum	PPB (pH 7.0)	0	0	83	83	92	92	92	94	94	30	4^{th} trifoliate	-
		PPB+2-ME	0	60	92	92	93	93	94	95	100	20	2^{nd} trifoliate	-
		PPB+SS	0	0	92	92	92	94	95	95	100	25	3^{rd} trifoliate	-
		PPB+SS+2-ME	0	83	95	95	100	100	100	100	100	20	2^{nd} trifoliate	-
		SE(m)	0.00	0.05	0.71	0.63	0.70	1.07	0.99	0.89	0.93			
		SE(d)	0.00	3.15	5.95	5.41	5.23	5.32	5.33	5.25	5.55			
		CD (0.01)*	0.00	0.19	2.68	2.35	2.64	4.03	3.73	3.36	3.48			

Note: PPB: Potassium Phosphate buffer; 2-ME: 2-mercaptoethanol; SS: Sodium sulfite; *Significant differences were obtained at P=0.01.

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 virus. Therefore, in the present investigation for maintenance of Urdbean leaf crinkle virus (ULCV) isolate, we have followed the buffer composition as described by Biswas *et al.* (2012) with modifications in inoculation method with addition of additives and abrasives.

Symptom observation

The urdbean leaf crinkle virus inoculated plants maintained in the glasshouse were observed up to 55 DPI. The inoculated plants showed similar symptoms as recorded earlier in the field conditions. Typical symptoms like crinkling of lamina, malformation of floral organs, stunting of the plant was observed (Fig 1B, D). Enlargement and rugosity of lamina was more prominent in the glasshouse developed symptoms when compared to field conditions (Fig 1C). Most of the inoculated plants did not show flowering up to 45DPI.

Validation of inoculation method

The optimized protocol was further evaluated on 9 urdbean genotypes which recorded per cent transmission ranging from 92-100% (Table 2). Amongst all the genotypes tested,

Table 2: Validation of optimized protocol.

Genotype	Transmission % at different day post inoculation (DPI) stages based on symptoms									Days required (DPI)	Stage of the crop at which symptoms expressed	Healthy control
	14	20	25	30	35	40	45	50	55			
VBN 4	0	0	0	0	21	32	42	42	42	35	4th trifoliate	-
VBN 5	0	0	0	15	32	32	53	53	53	30	3rd trifoliate	-
VBN 6	0	0	0	21	32	32	48	48	48	30	3rd trifoliate	-
VBN 9	0	0	0	32	36	42	56	56	56	30	3rd trifoliate	-
VBN 10	0	0	0	53	53	64	72	72	82	30	3rd trifoliate	-
VBN 11	0	38	42	42	53	72	85	92	92	20	2nd trifoliate	-
MDU 1	0	0	0	52	56	56	78	78	78	30	3rd trifoliate	-
CO6	0	0	0	32	53	72	82	82	82	30	3rd trifoliate	-
CO 5	0	42	48	56	56	74	100	100	100	20	2nd trifoliate	-
SEm	0.00	0.04	0.29	0.89	0.78	1.10	1.26	1.41	1.35			
SED	0.00	5.89	6.63	6.37	4.43	6.17	6.53	6.81	6.96			
CD (0.01)*	0.00	0.15	1.19	3.61	3.17	4.46	5.13	5.73	5.48			

*Significant differences were obtained at P = 0.01.

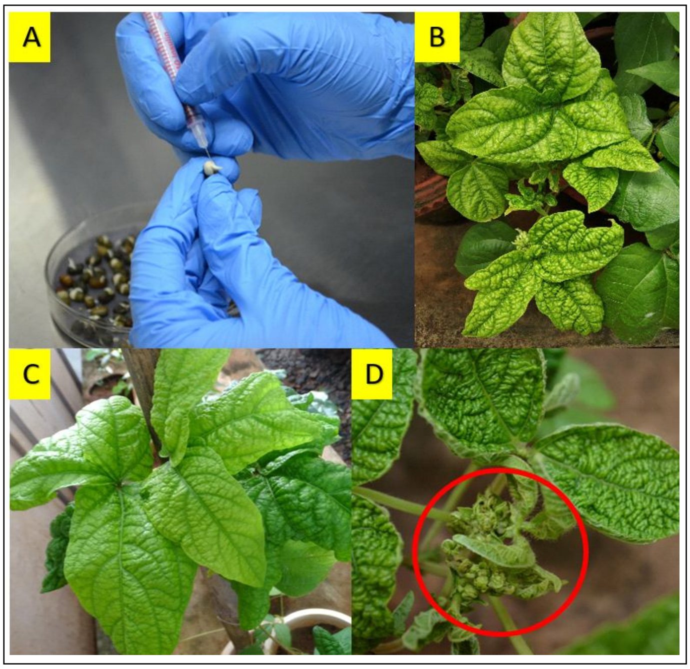

Fig 1: Symptoms developed by Sprout seed abrasion method. (A): Pin pricking of sprouted seeds; (B) Crinkling of lamina; (C): Enlargement of lamina; (D): Malformation of floral buds.

CO5 cultivar showed higher (42-100%) transmissibility 20 DPI *i.e.*, at 2nd trifoliate stage followed by VBN11, VBN 10 and CO6 (38-92%; 53-82%; 32-82%) respectively. Besides symptomatology, substantial changes in ULCD transmission percent were observed among the cultivars, demonstrating the pathogen preference and efficacy of the methodology. The authenticity and reproducibility of the methodology was further confirmed and it can be used for maintenance of virus inoculum as well as for screening of resistant genotypes against ULCD both under field and glasshouse conditions.

CONCLUSION

Evaluation of several factors affecting the sap transmission of ULCD resulted in development of a high throughput methodology for inoculation of ULCV which allows rapid transmission of large number of seedlings by immersing the sprouted seed in the virus suspension and expression of symptoms at the earliest. The standardized protocol will be highly useful for the maintenance of virus isolate under vector free conditions as well as for rapid evaluation of large number of urdbean cultivars for the source of ULCD resistance both under glasshouse and miniplot conditions. Further research is being conducted to determine the complete etiology of ULCD employing NGS techniques which will help to develop diagnostic techniques and to devise better disease management strategy.

Conflict of interest: None.

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