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Standardization of inoculation method for the maintenance of pure inoculum of leaf crinkle virus in Urdbean (*Vigna mungo* L. Hepper)

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Abstract
Urdbean or blackgram (*Vigna mungo* (L.) Hepper) is one of the important leguminous crops cultivated in India, Pakistan and South-East Asian countries. Among the biotic stresses, Urdbean leaf crinkle disease (ULCD) is economically a significant one and devastating the crop in major urdbean cultivating countries. ULCD causes severe crinkling and rugosity of lamina, stunting of plants, malformation of buds inflicting heavy yield losses. Due to occurrence of ULCD as mixed infection along with other major viruses infecting urdbean, maintenance of pure inoculum under glasshouse conditions is a prerequisite for the etiology confirmation work. In the present study, seeds were mechanically inoculated by sprout seed abrasion method as well as on to 2-leaf stage through infected sap. Influence of artificial inoculation on ULCD incidence has been studied in VBN 8 cultivar of blackgram. Symptoms started appearing 20 DPI and disease incidence was up to 88% in sprout seed abrasion method and 84% in sap inoculation at two leaf stage. The protocol mentioned in the present study helps in maintenance of pure inoculum of virus under glasshouse conditions and further investigations proved sprout seed abrasive method as efficient protocol to obtain transmission at higher levels at the earliest.

Keywords: Mechanical sap inoculation, sprout seed, urdbean, leaf crinkle, pure inoculum

Introduction
Urdbean (*Vigna mungo* (L). Hepper) or Blackgram is one of the most predominant cultivated crops of '*Vigna*' group belonging to 'Leguminosae' family. India is the primary origin of Urdbean whereas, Central Asia as secondary center (Vavilov, 1926) [17] and is cultivated from ancient times. This food legume is a good source of protein, minerals and energy. Urdbean is highly priced pulse, rich in phosphoric acid and very nutritious as it contains high levels of protein (25g/100g), potassium (983 mg/100g), calcium (138 mg/100g), iron (7.57 mg/100g), niacin (1.447 mg/100g), Thiamine (0.273 mg/100g), and riboflavin (0.254 mg/100g) (United States Department of Agriculture, Wikipedia).

The major urdbean producing states are Madhya Pradesh (39%), Rajasthan (15%), Andhra Pradesh (11%), Uttar Pradesh (9%), Tamil Nadu (9%), Jharkhand (4%) and Maharashtra (4%) computing for 91 per cent of total urdbean of the country. The production of urdbean is 2.43 million tonnes in India (1st advance estimates, Ministry of Agriculture and Farmers welfare, 2019-20). The disease was first reported in India by Nariani (1960) [12] from Delhi and in Tamil Nadu it was reported by Narayanasamy and Jaganathan (1973) [11]. The Urdbean leaf crinkle disease (ULCD) is 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, 1971; Subbarao, 1984; Kolte and Nene 1972; Ravinder Reddy 2005; Brar and Rataul, 1987) [18, 13, 10, 9, 15, 14, 6, 7]. Research on epidemiological aspects also explains that Urdbean leaf crinkle virus (ULCV) disease incidence depends upon the host genotypes, growing seasons and suitable environmental conditions (Ashfaq *et al.*, 2008) [2]. Certain resistant genotypes are now available to the breeders and farmers (Ashfaq *et al.*, 2008) [2] but no information is available on the mechanism of disease resistance in these germplasms. The yield losses may go up to 76 to 100% based up on the stage of crop infected (Brar and Rataul, 1987 and Bashir *et al.*, 1991) [7, 4].

Earlier research workers (Williams *et al.*, 1968; Chohan and Kalia, 1967) [18, 8] efforts failed to transmit ULCD through sap inoculation.

Later, Kolte (1972) [10] succeeded in the mechanical transmission of the causal virus using potassium phosphate buffer (PPB) (pH 7.6) as extracting medium and carborundum as abrasive. The incubation period ranged from 11-16 days in the plant for symptom expression. As various authors reported differently regarding the efficacy of ULCV 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.

Material and Methods

The experiment was carried out in the glass house of Department of Plant Pathology of Tamil Nadu Agricultural University (TNAU), Coimbatore.

Source of Inoculum

In order to maintain pure inoculum, Urdbean plants showing typical symptoms of ULCD from experimental fields of Tamil Nadu Agricultural University, Coimbatore were tagged at different stages of the crop and seeds were collected at harvest. Those seeds collected infected plants were sown in glasshouse under insect proof cages. Leaves showing typical leaf crinkle symptoms were collected from glasshouse sown plants and used as inoculum for further inoculation studies.

Inoculation by sprout seed abrasion method

Seeds of Urdbean cultivar VBN 8 was immersed in distilled water for 6 hours for for the sprouts to emerge, pre-soaked seeds were placed on moist blotter paper for 8 hours (Biswas *et al.*, 2012) [5]. 1g of infected leaf sample was ground in 5ml of potassium phosphate buffer (0.05M, pH 7.0) and supplemented with 0.1 percent 2-Mercaptoethanol in a sterilised and chilled ice-cold mortar and pestle (Fig 1). (0.05M 2.335g of Dipotassium hydrogen phosphate in 500ml of sterile distilled water was used as Sol A, while 1.575g of Potassium dihydrogen orthophosphate in 500ml of sterile distilled water was used as Sol B. To achieve the desired combination and pH, 8.9ml of Sol A and 61.1ml of Sol B are combined.

Sprouted seeds were incubated in the sap along with carborundum as abrasive. Seeds were soaked for one hour with intermittent shaking and sown in pots (dia. 20") with nutrient supplement, timely irrigation and kept under insect proof cages. Incubating sprouted seeds with buffer was maintained as control.

Fig 1: Sprout seed abrasion methodology on urdbean plants under Insect proof condition

Mechanical transmission through sap inoculation

ULCD infected symptomatic leaves were collected and macerated with 0.05 M PPB (pH 7.0) and 0.1% 2-Mercapto ethanol for mechanical transmission through infected leaf sap. Urdbean cv. VBN 8 was sown in pots under an insect-proof glasshouse and infected leaf sap was inoculated with carborundum as an abrasive at the two-leaf stage of the crop (Fig 2). After inoculation with virus suspension leaves are washed with distilled water to remove excess inoculum. Similar to sprout seed inoculated plants, these plants were also maintained separately under insect proof screen house conditions along with uninoculated control.

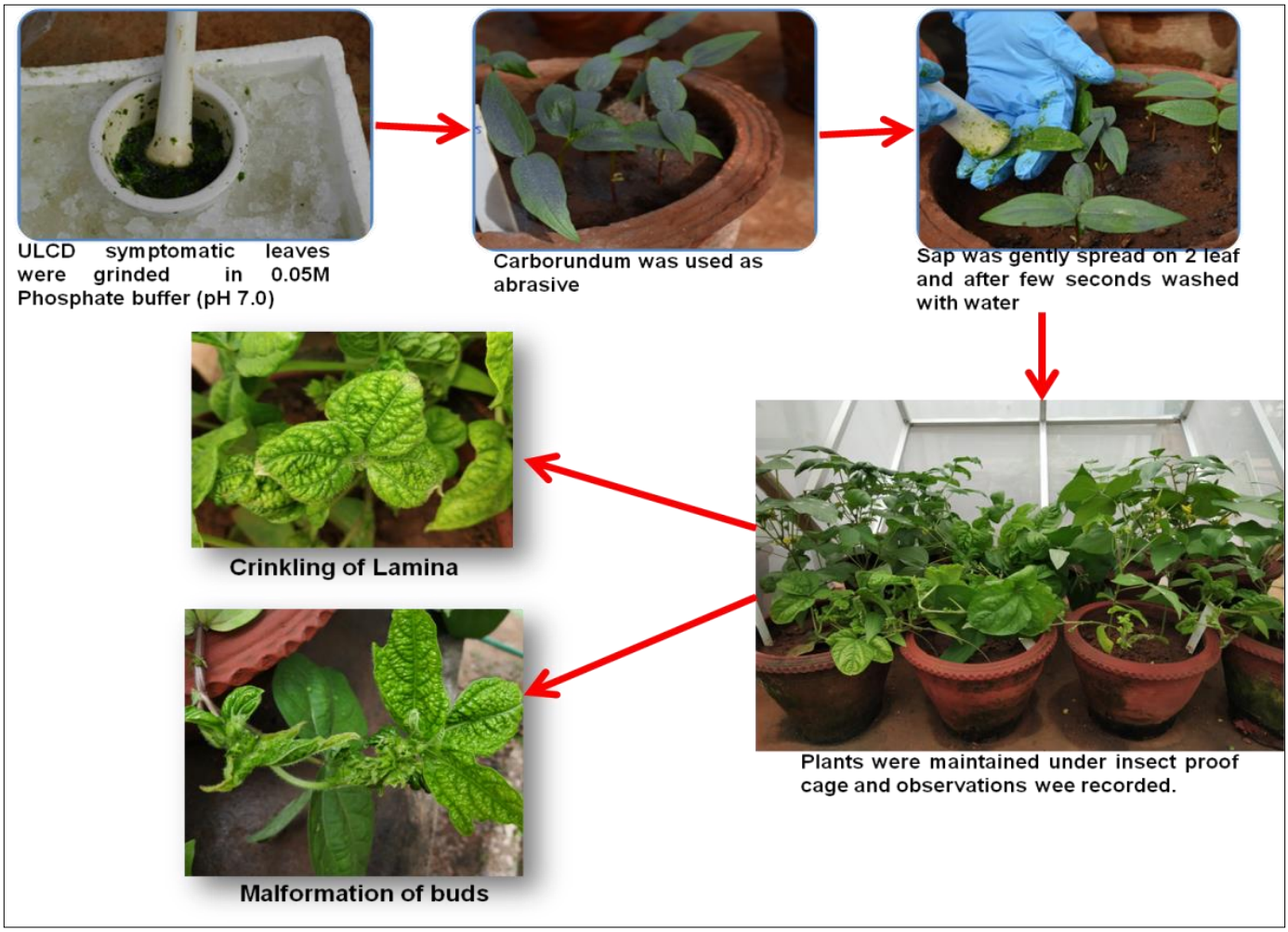

Fig 2: Mechanical sap Inoculation methodology on urdbean plants under Insect proof condition

Observation on ULCD Incidence

Symptom development was observed starting from 20 days post inoculation (DPI) for both the methods. Number of seedlings showing typical leaf crinkle symptoms were observed (Fig 3) and per cent disease incidence was recorded up to 50 DPI. Typical symptoms *viz.*, crinkling and enlargement of lamina, malformation of buds was observed. Per cent disease incidence (PDI) was calculated as:

$$\text{Per cent disease incidence} = \frac{\text{Number of infected plants}}{\text{Total number of plants assessed}} \times 100$$

Results and Discussion

Sprout seed inoculated as well as sap inoculated urdbean plants were maintained under insect proof cage separately along with uninoculated control were observed at regular intervals and observations were recorded as per cent Disease incidence (Table 1). Symptoms started as mild crinkling of lamina from 20 DPI in case of sprout seed abrasion method and from 25 DPI in sap inoculated plants. Symptoms in both methods progressed as extreme crinkling and puckering followed by enlargement, rugosity of lamina and malformation of floral buds. No symptom was observed on uninoculated plants.

Observations were recorded and per cent disease incidence was noted in regular intervals for both the methods. Among both the protocols, sprout seed abrasion method showed higher transmission of 88%, when compared to normal sap inoculation method (84%). The symptom appeared earlier in the sprout method at 20 DPI (2nd trifoliate stage), which could be attributable to the inoculation timing and stage, which is done as early as possible to the meristem (sprouted seed).

Table 1: Comparison of inoculation method for urdbean leaf crinkle disease incidence

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

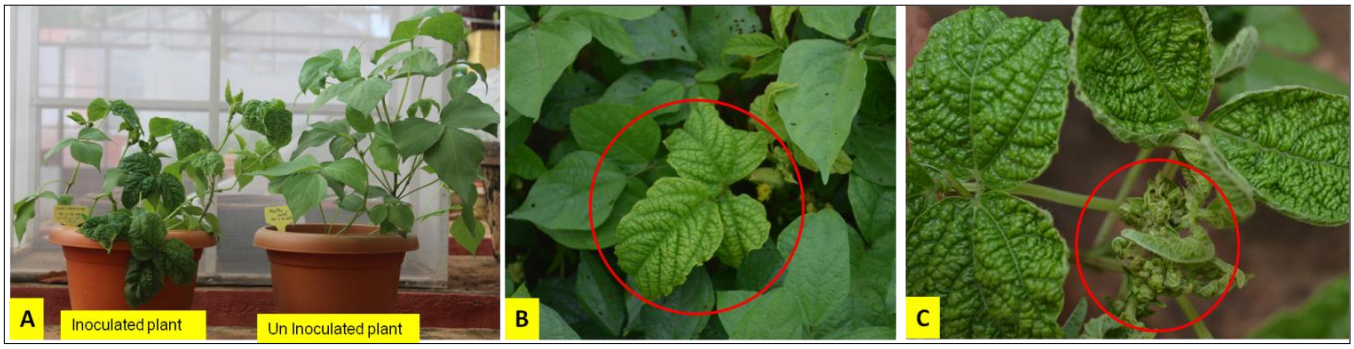

Fig 3: Symptom development in inoculated plants; A: Inoculated and Uninoculated plants; B: Crinkling of Lamina; C: Malformation of floral parts

Conclusions

In the present study seeds were mechanically sap inoculated by sprout seed abrasion method as well as on to 2-leaf stage. Influence of artificial inoculation on ULCD incidence has been studied on VBN 8 cultivar. Biswas *et al.*, (2012) [5] also used sprout seed abrasion method to evaluate resistant sources of different urdbean genotypes against urdbean leaf crinkle virus. Baranwal *et al.* (2020) made efforts to inoculate ULCV at different growth stages of urdbean plant and reported 100% disease severity when inoculation was done to sprouted seeds and incidence was reduced when inoculated to first trifoliate and subsequently. 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 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 urdbean leaf crinkle disease is still unknown, maintaining a pure inoculum will help in eliminating the mixed infections and investigating host pathogen interactions.

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