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Research Article

Standardization of Agroinoculation Technique for Mungbean Yellow Mosaic Virus (MYMV) Infecting Black Gram (*Vigna mungo* L. Hepper)

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ABSTRACT

An efficient procedure for sprouted seed method of agroinoculation in black gram crop with infectious dimer agro clones of mungbean yellow mosaic virus (MYMV) DNA-A and DNA-B has been developed using black gram susceptible genotype PBG-1. Different parameters like Agrobacterium cell density, concentration of acetosyringone, inoculation buffer, incubation time, incubation temperature and place of pinpricking were standardized for maximum infectivity of MYMV virus in agroinoculated plants. The Agrobacterium cell density at OD_{600} value 0.8 gave high percentage of virus infection followed by OD_{600} value 1 and 0.5. It was observed that high percentage of virus infection gave in 100 µm concentration of acetosyringone followed by 80 µm and 150 µm concentrations of acetosyringone. Agroinoculated blackgram seeds were incubated at three incubation times (1hour, 5hours and overnight) and 80-100% percentage of infection was observed at all three incubation times, similarly incubation temperatures (25°C, 28°C and 37°C were evaluated for maximum percentage of infection and 82% of infection was observed at incubation temperature 28°C followed by 25°C and37°C, even poor germination was noticed at incubation temperature 37°C. First time we evaluated the place of pinpricking on hypocotyl region for maximum virus infection and found that pinpricking around the hypocotyls gave high percentage of virus infection (79%) in agroinoculated blackgram plants.

Key words: *Sprout seed method of agroinoculation, Parameters and MYMV.*

INTRODUCTION

Blackgram (*Vigna mungo* (L) Hepper) is one of the major pulse crops of the tropics and sub tropics. It is the third major pulse crop cultivated in the India. Yellow mosaic disease (YMD) is the major constraint to the productivity of grain legumes across the Indian subcontinent[11]. The disease in South Asia is caused by four distinct begomoviruses collectively known as the yellow mosaic viruses (YMVs); Mungbean yellow mosaic virus (MYMV), Mungbean yellow mosaic

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India virus (MYMIV), Dolichos yellow mosaic virus (DoYMV) and Horse gram yellow mosaic virus (HgYMV). Of these MYMIV and MYMV are most important as it infect large number of legumes in India. MYMIV is more predominant in northern, central and eastern regions of India[9] and MYMV in southern region[6] to which Andhra Pradesh state belongs. Reddy *et al.*[8] reported the existence of two species of begomoviruses (MYMIV &MYMV) with YMD of black gram in Andhra Pradesh and concomitant existence of both species results in variation of symptoms in black gram. It is also difficult to confirm the resistance to which species of begomovirus under field conditions. Hence screening of black gram germplasm against YMV disease is difficult due to mixed infection of MYMV and MYMIV under field conditions. Hence, plant breeders and pathologists are in need of a biological /molecular tool that can lead to the identification of MYMV & MYMIV resistant /susceptible genotypes. In this context agroinoculation is a useful method by which germplasm can be screened against particular species of virus or strain of virus under green house conditions[1]. Previous researchers working on urdbean, mungbean and soybean[4,9,2,7] demonstrated the feasibility of using an *in vitro* agroinoculation procedure in MYMV studies.

In this study we focused on the standardization of agroinoculation (Sprouted seed method) in Black gram. Different parameters which affect the agroinoculation were studied for efficient agroinoculation.

MATERIAL AND METHODS

Blackgram genotype:

Susceptible blackgram genotype was used in this study, namely LBG-645 for standardization of sprouted seed method of agroinoculation for large scale screening of blackgram genotypes against Yellow mosaic disease.

Infectious *Agrobacterium* dimer clone of MYMV DNA-A &DNA-B:

The infectious dimer clones of MYMV DNA-A and MYMV DNA-B in pCAMBIA2301 vector were constructed in *Escherichia coli*, then mobilized in to *Agrobacterium tumefaciens* EHA 105 cells by freez -thaw method and used in this study for agroinoculation.

Agroinoculation Procedure:

EHA 105 *Agrobacterium* cells harboring full length dimers of MYMV DNA-A & DNA-B were grown to optimum growth stage and mixed in equal proportion. Bacterial cells were collected by low speed centrifugation and cells were resuspended in small volume of broth with required concentration of acetosyringone and used for inoculation. Seeds of blackgram plants were surface sterilized and soaked in sterile water for 2-3hrs and kept for germination overnight at room temperature, seed coat of sprouted seeds were removed by using forceps and pinpricked with fine needle, and were immediately immersed in *Agrobacterium* cells containing MYMV DNA-A and DNA-B. After optimum incubation time, seeds were washed and sown in pots. These pots were maintained in an insect-free plant growth chamber at 25±2oC, 60-70% RH with a 16 hr photoperiod.

Standardization of different parameters for efficient agroinoculation:

Different parameters like *Agrobacterium* cell density/bacterial growth stage, concentration of acetosyringone, incubation time, incubation temperature and place of pinpricking were standardized for sprout seed method of agroinoculation for maximum infectivity of MYMV virus in agroinoculated plants.

RESULTS AND DISCUSSION

I. Standardization of different parameters for efficient agroinoculation:

1. Effect of *Agrobacterium* growth stage on agroinoculation:

In order to determine the optimum growth stage of *Agrobacterium*, different growth stages of *Agrobacterium* inoculums (OD_{600} values of 0.5, 0.8 and 1) were used for agroinoculation with sprouted seeds of blackgram susceptible genotype. The *Agrobacterium* culture at their optimum growth stage gave maximum infectivity of

virus in agroinoculated plants. For this we also compared three types of broths with neutral pH (Agrobacterium minimal broth, yeast extract peptone broth and Luria broth) for fast growth of *Agrobacterium* clones. Among three types of broth, Agrobacterium minimal broth gave high optical density within a short time compared to other two broths followed by Luria broth and yeast extract peptone broth (Table.1). Data on Percentage of YMV infection was taken in agroinoculated plants with three growth stages of *Agrobacterium*. The characteristic yellow mosaic symptoms were observed on first emerged trifoliate leaf of agroinoculated susceptible blackgram genotype at 12 days after sowing (DAS). Results indicated that of all three media used here, at OD_{600} value 0.8 gave high viral infection of 65% followed by OD_{600} values at 1 and 0.5 with viral infection of 63% & 59% respectively. Jacob *et al*.[4] found Agrobacterium cultures containing partial tandem repeats of MYMV to an optical density of 1 at 600nm is ideal for agroinoculation method in blackgram crop, But in our study Agrobacterium culture at OD_{600} value 0.8 gave high viral infection. OD_{600} value 0.8-1 represents active logarithmic phase of bacteria which is suitable for transformation.

Table1: Effect of *Agrobacterium* growth stage on agroinoculation in Blackgram

S.No	Media used for *Agrobacterium* growth	OD 600nm	Time taken for reaching OD values	% of Infection	Time taken for expressing symptom
1	AB Minimal Media	0.5	15hours	36	8-12 DAS
		0.8	20hours	65	
		1	28hours	52	
2	Yeast Extract Peptone Media	0.5	19hours	28	
		0.8	23hours	59	
		1	35hours	48	
3	Luria Broth	0.5	18hours	31	
		0.8	22hours	63	
		1	32hours	49	

2. Effect of Acetosyringone concentration:

Experiments were carried out to evaluate the effect of different concentrations of acetosyringone on agroinoculation for maximum infectivity of MYMV. For this three concentrations of acetosyringone (80µm, 100 µm and 150 µm) were tested. It was observed that high percentage of virus infection gave in 100 µm (71%) concentration of acetosyringone followed by 80 µm (59%) and 150 µm (47%) concentrations of acetosyringone. These results were similar to Jacob *et al.*[4] reported that 100 µm concentration of acetosyringone is ideal for maximum infectivity. In our study we noticed that presoaking of pinpricked blackgram seeds in100µm acetosyringone solution for half an hour before adding this into AB minimal broth containing MYMV constructs gave maximum percentage of infection (80%).

3. Effect of incubation time and temperature:

In order to determine optimum incubation time for pinpricked blackgram seeds in *Agrobacterium* culture containing MYMV DNA-A &DNA-B dimers, we compared three incubation timings *i.e* 2hours, 5 hours and overnight for maximum virus infectivity. The more or less equal percentage of YMV infection (80-100%) was observed at all three incubation times. Agroinoculation procedure was highly depends on temperature. For confirming this, agroinoculated blackgram seeds were kept in three different temperatures at 25^{o}C, 28^{o}C and 37^{o}C. These three temperatures were tested /compared with overnight incubation. The maximum percentage of YMV infection (82%) gave at 28^{o}C followed by 25^{o}C (78%) and 37^{o}C (61%) of incubation temperature, where as the

germination percentage of agroinoculated plants was poor at 37°C incubation temperature. Mandal *et al.*[5] and Jacob *et al.*[4] reported that the pinpricked seeds were kept in Agrobacterium cultures containing MYMV constructs at 28°C overnight. In our study we observed that even with 2 hours of incubation period at 28°C of incubation temperature gave similar percentage of infection compared to overnight incubation. This experiment clearly suggest that percentage of YMV infection was good at its shortest incubation period (2hours) instead of overnight incubation as it saves time and improves germination percentage of pinpricked seeds in overnight incubation was low compared to 2hours incubation period.

5. Effect of place of pinpricking on hypocotyls region of blackgram seed:

In order to get maximum virus infection pinpricking was tested in two ways on hypocotyls region of blackgram cotyledons. In case of first case pinpricking was done around the hypocotyls region, while in a second case pinpricking was done along the hypocotyl region of blackgram cotyledons. The maximum percentage of YMV infection was observed in seeds prinpricked around hypocotyls (79%) as compared to other method (40%).

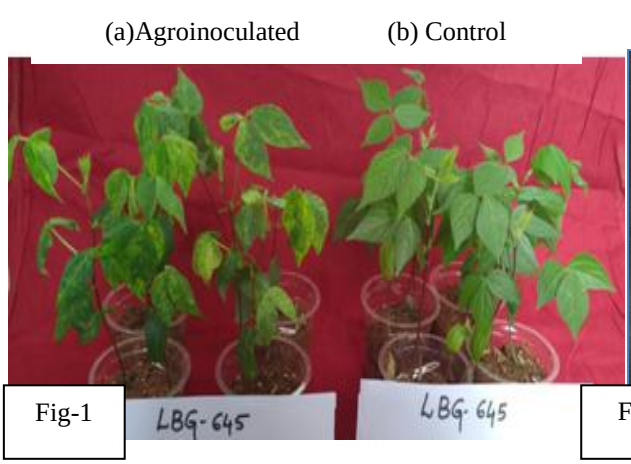

Fig-1

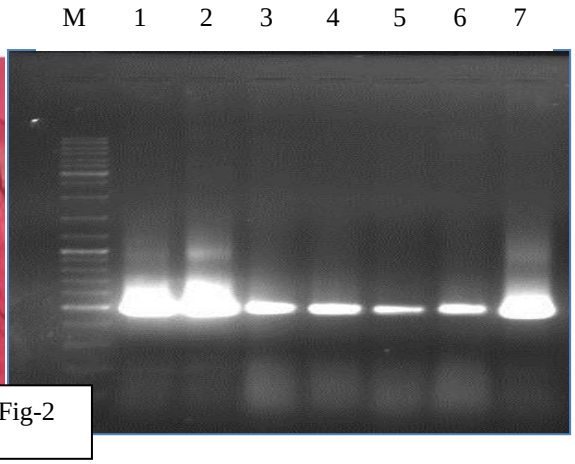

Fig-2

Fig. 1: A: Yellow mosaic symptoms upon agroinoculation with dimers of MYMV DNA-A &MYMV DNA-B at 30DAS B: Detection of MYMV-DNA-A in Agroinoculated and control Blackgram plants.

Fig. 2: Lane M: 1KB ladder SMO 331. Lane2-3: Agroinoculated symptomatic plants. Lane4-5: Agroinoculated asymptomatic plants. Lane 5-6: Control blackgram plants.

Finally, agroinoculation procedure was conducted with all standardized parameters by using two susceptible checks (PBG-1 & LBG-645). The characteristic yellow mosaic symptoms were observed from 8-12 DAS (Fig 1). The percentage of viral infection was calculated and it was 95% in PBG-1, 85% in LBG-645 susceptible check. The symptomatic, asymptomatic agroinoculated plants and control plants were screened by polymerase chain reaction with MYMV specific primers.

The PCR results showed the presence of MYMV virus in both symptomatic and asymptomatic agroinoculated blackgram plants, even in control plants also virus presence was noticed, but intensity of band with agroinoculated plant DNA was observed as high compared to control plant DNA (Fig 2). That means the concentration of virus in control plants were low compared to agroinoculated plants. These results clearly indicated the seed borne nature of the YMV virus[10]. It is well known that the presence of a virus in a seed, even in the embryo does not always lead to seedling infection[3].

CONCLUSION

The Agrobacterium mediated virus inoculation method (sprouted seed method of agroinoculation) for screening of Yellow mosaic disease resistance was standardized and the various factors that affect the efficiency of agroinoculation were examined. A standard protocol was developed for sprouted seed method of agroinoculation in back gram crop which includes inoculation of sprouted seedlings in *Agrobacterium* suspension having OD_{600} value of 0.8 by pinpricking around the hypocotyl region of sprouted black gram seed and incubated in Agrobacterium culture containing MYMV DNA-A & DNA-B constructs at 28^{o}C for 2 hours of incubation period with 100 µm acetosyringone showed high percentage of MYMV infection in agroinoculated plants, which could be useful to screen large scale blackgram germplasm for their resistance against yellow mosaic disease.

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