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Evaluation of Inoculation Methods and Standardization of *Erwinia chrysanthemi* Inoculum Concentration for Germplasm Screening against Stalk Rot in Sorghum

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Stalk rot caused by *Erwinia chrysanthemi (Ech)* is one of the most destructive diseases of sorghum crop. The bacterium was isolated from infected sorghum plants collected from livestock research centre, G.B. Pant University of Agriculture and Technology, Pantnagar, India. Evaluation of inoculation methods viz. leaf whorl inoculation, stem injection method, root tip cut dip method and tooth pick method was done in glasshouse by inoculation from 24 h old culture of *Ech* adjusted to 1×10^6 cfu/ml, 1×10^7 cfu/ml and 1×10^8 cfu/ml by adding sterilized distilled water and 0.7% (v/v) of tween-40 (surfactant). Leaf whorl inoculation, stem injection and root tip cut and dip method which showed significant results in glass house were further used for field experimentation. Root tip cut and dip method was observed to be the best inoculation method both in glasshouse and field experimentation and the optimum infection was reported at 1×10^7 cells/ml (cfu).

Keywords: Stalk rot, *Erwinia chrysanthemi*, leaf whorl inoculation, stem injection method, root tip cut dip method, tooth pick method.

Stalk rot of sorghum caused by *Erwinia chrysanthemi* Burkholder, McFadden, and Dimock is one of the most destructive diseases of sorghum crop. Saxena *et al*. (1991); reported this bacterium causing stalk and top rot of sorghum under natural conditions in India during 1987-88 crop season in sorghum field at Pantnagar, Uttarakhand. The disease was wide spread and affected 60-80% of plants in different sorghum genotypes. The infected stem pith is disintegrated and show slimy soft-rot symptoms with foul-smell and eventually the whole plant wilts (Zummo, 1969; Hseu *et al*., 2008; Hepperly and Ramos-Davila, 1987). The rot may involve only one or two internodes, or the entire length of the stalk, which finally dries up and its interior turns into a shredded mass of fibrous tissue. Lower leaves and leaf sheaths covering the internodes are chlorotic, and the rind is pale-straw instead of green in colour. The economic, biomass and grain yield losses due to rapid progress of this bacterial soft rot disease is one of the most destructive feature in natural condition. The disease appears before the onset of flowering. Cloudy weather, relatively high temperature (>30° C) and frequent rainfall favors disease epidemic (Saxena *et al*., 1991).

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MATERIALS AND METHODS

On the basis of visual observation, infected plants with typical soft rot symptoms were collected from livestock research centre, G.B. Pant University of Agriculture and Technology, Pantnagar in the growing season 2014-2015. Samples were kept in polythene bags under refrigerated condition at 4°C. The experiment was

conducted at Centre of Advanced faculty training in Plant Pathology, Department of Plant Pathology, College of Agriculture, G.B. Pant University of Agriculture and Technology, Pantnagar, Uttarakhand, India.

Isolation and purification of the isolate

Isolation of bacterium was done as per the method described by Janse (2005). Pieces of tissue taken from the margin of healthy and diseased tissues were disinfected with 70% alcohol and placed in a sealed tube with sterile water. Tissues were left for 30 min in suspension so that the bacteria could diffuse out of the tissues. Subsequently 100 µl of the suspension was plated onto crystal violet sodium polypectate (CVP) medium. Characteristically deep-pit forming colony on CVP medium purified on yeast dextrose calcium carbonate medium by streaking using freshly growing single colony and these plates were incubated at 28°C for five days. The isolate was preserved in NA slants at 4°C.

Pathogenicity test

To confirm the pathogenicity of isolate, leaf whorl inoculation was done on 21 days old plants of susceptible sorghum cultivar under controlled glasshouse conditions. Isolate was grown on Luria Broth for 24 h at 28°C. The bacterial cells were suspended in sterile distilled water and the cell density adjusted to 1×10^7cfu/ml. Bacterial suspension [0.7 % Tween-40 (v/v) + 1×10^7 cfu/ml] of isolate was inoculated in leaf whorl with the help of atomizer . The control was sprayed with sterilized water. Experiment was conducted thrice to confirm the result.

Preparation of inoculum on Luria broth

The test bacterium *E. chrysanthemi* isolated and purified from fresh diseased stalk of sorghum was used throughout the investigation. For preparation of inoculum Luria broth medium was used. Single typical colony of *E. chrysanthemi* was inoculated in each flask aseptically and then flasks were incubated at 28± 1°C for 24 hrs. The flasks were incubated on shaker incubator for uniform bacterial growth on broth then used as inoculum for artificial inoculation in glasshouse and field experiments.

Glasshouse experiment

Experiment was conducted in glasshouse using healthy seeds of susceptible sorghum cultivar SPV 2128. Ten seeds were sown in 30 cm plastic pots filled with sterilized soil. As to obtain 21 days old seedlings for inoculation, these pots were kept in glasshouse and irrigated with water regularly to maintain high moisture conditions. Before inoculation only 5 seedlings per pot were maintained, rest were uprooted. Bacterial cell suspension was prepared from 24 h old culture of *Ech* and adjusted to 1×10^6 cfu/ml, 1×10^7 cfu/ml and 1×10^8 cfu/ml by adding sterilized distilled water and 0.7% (v/v) of tween-40 (surfactant). Twenty one days old plants were inoculated with this bacterial suspension between 5-7 pm, by four different methods viz. leaf whorl inoculation, stem injection, tooth-pick and root tip cut and dip. For control only sterilized water was used. Immediately after inoculation plants were placed in moist chamber for 48-72 hours and then transferred in glasshouse having a temperature of about 30 ± 1^0C and relative humidity > 90%. The symptoms expressed were studied and re-isolation of the pathogen was made. Experiment was conducted using completely randomized design (CRD) with three replications. The inoculation test, as above was repeated once more to confirm the result. Disease assessment was done based on percentage of plants showing stalk rot symptoms in relation to total inoculated plants after one week of inoculation (Hartman and Kelman, 1973). Three parameters viz., percent lodging, number of internodes crossed and length of spread lesion (observed visually after split-open the infected stalks) by the *Ech* were used.

Inoculation techniques

Leaf–whorl inoculation method

Leaf-whorl inoculation method was adopted from Hartman and Kelman (1973) used in corn inoculation of *Erwinia* spp. without causing injury. Bacterial suspension was sprayed in leaf whorls (2ml/whorl) with the help of atomizer without causing any injury. Care was taken not to disturb the plants after inoculation so that maximum inoculum was retained in the leaf whorls. Plants sprayed with sterilized water served as control. Leaf whorl inoculation technique was used successfully in sorghum plant (Hepperly *et al*., 1987).

Stem injection method

Bacterial suspension was inject-inoculated with a 21 G hypodermic needle into the vicinity of a growing point of 21 days old

susceptible plants as described by earlier investigators (Thind and Payak, 1978; Aysan *et al*., 2005; Ruz *et al*., 2008; Kutama *et al.,* 2011). Control plants were inject-inoculated with sterilized water only.

Root tip cut and Dip method

Root tip cut and Dip method was adopted from Bolick (1960) used to prove pathogenicity of *Erwinia chrysanthemi* in chrysanthemum causing bacterial bud blight. Twenty-one days old plants grown in nursery were watered well 1 h before lifting the plants. Care was taken, not to damage the root and crown portion while uprooting the plants. The adhered soil to the roots was washed gently in tap water followed by washing in sterilized water to avoid the undesirable soil borne microbes or pathogens. The roots were cut off about 3 cm from the tip portion of main or primary root and immediately dipped in 250 ml beaker containing 100 ml of bacterial suspension; the plants were transplanted immediately into pots filled with sterilized, well watered soil in glasshouse. The roots of plants used for control were dipped in sterilized water.

Tooth–pick method

Sorghum stalks were inoculated with *Ech* using the wooden toothpick method of inoculation as described by Young (1943); Crall (1952); Hildebrand (1953) and Clements *et al*., (2003). Toothpicks were boiled thoroughly in water for two hours to remove resin, gum or any other toxic substances that might inhibit the growth of *Ech*. After boiling, they were washed thoroughly in tap water, and then toothpicks were dried under the sun. About 10 toothpicks were placed in 100 ml flasks in such a way that the pointed end of toothpicks faced away from the base and were autoclaved at 15 pounds psi (temperature 121^0C) for 20 minutes. *Ech* was inoculated to 100 ml flasks containing sterilized LB, under aseptic condition, incubated at 28^0C and a rich suspension of bacterial cell was made within 7 days. This suspension was further poured into toothpick containing flasks to cover lower $1/3^{rd}$ of the toothpicks under aseptic conditions and flasks were incubated for seven days at 28^0C, by the time toothpicks were covered with bacterial growth and were ready for inoculation. To confirm that toothpicks were colonized by *Ech*, infested toothpicks were streaked onto Petri plates amended with NGM and growth with blue pigment was observed. A sterile pointed iron needle (1-2 mm diameter) with a wooden handle was used to make a hole in the stem, to facilitate toothpick insertion. Toothpicks were introduced obliquely into the stalk in 21 days old plants. The control plants were inoculated with a non-infested and sterilized toothpick. Care was taken not to insert the toothpick too deeply in order to avoid splitting of the stalk. Cares were taken to ensure that drought stress conditions prevailed at the time of toothpick insertion. The toothpick inoculation technique has been used to screen germplasm against sorghum and maize pathogens (Bramel-Cox and Claflin, 1989; Clements *et al*., 2003; Tesso *et al*., 2009; Sobowale, 2011).

Field experiment

Leaf whorl inoculation, stem injection and root tip cut and dip methods which showed significant results in glass house were further used for field experimentation. Twenty one days old plants were artificially inoculated during evening hours between 5-7 PM as night temperature and humidity are conducive for infection following the same methodology as followed in glasshouse experiment. The experiment was performed in randomised block design (RBD) with three replications.

Disease observation

Observations on severity of the disease were recorded in 0 to 5 scale modified and adapted from Muhammad (1983) used for evaluation of corn germplasm against Erwinia stalk rot as follows:

0 = No symptoms

1 = Initial small necrotic areas/ partial rotting at the base of the whorl/ stalk

2 = 25-49% dark brown, water soaked, soft or slimy at the base of the whorl, disintegration of the pith tissues at a single internode, premature wilting of uppermost leaves

3 = 50-74% decay spreading rapidly crossing 2-3 internodes in collapsed plant

4 = 75-100% of tissue rotted with foul smell at the base of whorl/extensive necrosis/soft rotting with visible external symptoms

5 = lodging accompanied by extensive necrosis/ rotting of leaf/ stalk tissue usually having a very strong foul smell

RESULTS AND DISCUSSION

All the four method used for artificial inoculation were found effective in glasshouse conditions in causing disease. In leaf whorl inoculated plants, symptoms appeared after 7 days of inoculation; as curling, yellowing and wilting of apical leaves. Red discolouration of main vein with brown necrotic leaf spots, later with a sunken necrotic centre and malformation of newly formed leaves was observed. Infected leaves on pulling separated easily from stalk at the point of bacterial rot. Finally the whole plant showed chlorosis and necrosis leading to death of plant. In stem injection, the rotting started at point of injection of bacterial suspension usually 5 days after inoculation and then it expanded in both the direction. Artificial inoculation by stem pricking method and leaf pricking method of inoculation with *Erwinia chrysanthemi*, has been found effective in symptom development of the bacterial stalk rot of sorghum (Hseu *et al.*, 2008). Abdullah (1982) reported that the whorl inoculation and sheath injection as effective method of inoculation for *Erwinia chrysanthemi* in corn. In root tip cut and dip method the symptoms appeared usually 3 days of inoculation as rotting of basal portion of stem and there was sudden collapse of entire plant. Symptoms similar to as observed under glass house conditions were observed in field. In glasshouse maximum disease severity was observed in root tip cut and dip method (93.51%) followed by stem injection (86.02%) and leaf whorl inoculation method (82.30%) at 1 x 10^8cfu/ml. Least disease severity at 1 x 10^8cfu/ml was observed with toothpick method (75.24%). Among the concentrations, 1 x 10^6cfu/ml was least effective in developing disease whereas 1 x 10^8 cfu/ml developed most severe symptoms. Similar result was observed in field experiment maximum disease severity was recorded in root tip cut and dip method (53.62%) followed by leaf whorl inoculation (44.62%) and stem injection (41.71%) methods at 1 x 10^8 cfu/ml. Among concentrations, 1 x 10^8 cfu/ml developed maximum disease severity for each inoculation method whereas 1 x 10^6 cfu/ml developed minimum disease severity. However, there was not much increase in disease severity as concentration increased from 1 x 10^7 cfu/ml to 1 x 10^8 cfu/ml as compared to, when the concentration was increased from 1 x 10^6 cfu/ml to 1 x 10^7 cfu/ml.

Hossain and Logan (1983) used two methods of inoculating potato tubers, one by dipping them in an aqueous suspension of *Erwinia*

Table 1. Evaluation of artificial inoculation methods and different bacterial concentration after 10 days of inoculation in glasshouse.

Artificial inoculation method	Bacterial cell conc. (cfu/ml)	Disease severity (%)
Leaf-whorl inoculation method	1 x 10^6	74.92
	1 x 10^7	81.45
	1 x 10^8	82.30
Stem injection method	1 x 10^6	80.60
	1 x 10^7	85.16
	1 x 10^8	86.02
Root tip cut and dip method	1 x 10^6	86.07
	1 x 10^7	91.30
	1 x 10^8	93.51
Tooth-pick method	1 x 10^6	69.36
	1 x 10^7	74.47
	1 x 10^8	75.24
CD at 5 %	Treatment a	0.97
	Treatment b	0.84
	a*b	1.69

Table 2. Evaluation of artificial inoculation methods and different bacterial concentration after 10 days of inoculation in field

Artificial inoculation methods	Bacterial cell conc. (cfu/ml)	Disease severity (%)
Leaf-whorl inoculation method	1 x 10^6	37.15
	1 x 10^7	42.95
	1 x 10^8	44.62
Stem injection method	1 x 10^6	35.53
	1 x 10^7	38.91
	1 x 10^8	41.71
Root tip cut and dip method	1 x 10^6	44.64
	1 x 10^7	50.50
	1 x 10^8	53.62
CD at 5 %	Treatment a	0.93
	Treatment b	0.93
	a*b	1.62

carotovora ssp. *atroseptica*, the other by inserting the end of a toothpick charged with undiluted bacteria to produce black leg disease. Hartman and Kelman (1973) recommended the leaf whorl inoculation in corn for inoculation of *Erwinia* spp. at ten different concentration ranging from 1 x 10^5 cells/ml to 1 x 10^8 cells/ml and found that the infection percentage increased upto 1 x 10^7 cells/ml but no significant increase in infection was observed after it with increase in bacterial concentration. Strider (1970) used root and stem inoculation method in tomato seedlings for inoculation of *Corynebacterium michiganense* of different cell concentration. Inoculation with bacterial suspension by leaf whorl inoculation and through hypodermic needle in vicinity of growing point has been used (Zummo, 1969; Jensen, 1986; Saxena *et al*., 1991). Tyner (1947) demonstrated that potato plant could be infected by dipping wounded root tips in bacterial suspension of *Corynebacterium sepedonicum*. Suspension of bacterium cells have also been infiltrated into tissues of maize plants and stems below the first leaf whorl with a syringe (Goszczynska *et al.*, 2007). Results showed varying extent of disease development by stem injection method that received different concentrations of test pathogen in sorghum and other crops by various investigators (Anderson and Gardner, 1999; Kutama *et al*., 2011; Sobowale, 2011).

CONCLUSION

Evaluation of different methods of artificial inoculation helped us to determine root tip cut and dip as the most suitable method which can be used for screening of sorghum germplasm against *Erwinia chrysanthemi* . It can also be used to screen the strains or isolates of the bacterium for virulence.

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