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Divulging the comparing inoculation methods for assessing pathogenicity of *Dickeya dadantii* inciting stalk rot disease of sorghum

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Abstract
Dickeya dadantii causes bacterial stalk rot of sorghum crop including Maize, a most devastating disease in arid and semi-arid tropics. Saxena *et al*. (1991), spelled this bacterium causing most destructive diseases of 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 disease mainly affects sorghum stem showing water-soaked symptoms that later turn reddish dark brown color. Stalk rot bacterial pathogen was isolated from diseased samples collected from livestock research centre, G.B. Pant University of Agriculture and Technology, Pantnagar. The pathogenicity test was proved on 21 days old sorghum plant in glasshouse. It was found that virulent pathogen developed symptoms with 3 days incubation period and maximum required seven days for development of disease. Preliminary symptoms appeared as water soaked to brown spot on leave and stalk. However, this study was conducted to find out efficient inoculation methods and measured the disease severity for *D. dadantii* on sorghum and replicability. Bacterial suspension (0.7 % Tween-40 + 2 x 10^8 cell/ml) was inoculated with different inoculation techniques, such as (1) Immersion of wounded roots in the bacterial cell suspension (2) Injection of bacterial cell suspension in the base of the stem (3) Leaf-Whorl & Spraying the inoculums by way of hand atomizer (4) Tooth –pick inoculation method (5) Midrib injection method (6) Cotton wool method. Highest stalk rot disease severity (89.75%) was investigated in case of immersion of wounded roots in the bacterial cell suspension method followed by others.

Keywords: Comparing inoculation, pathogenicity *Dickeya dadantii*

Introduction
Sorghum is cultivated widely throughout the arid and semi-arid tropics. Sorghum is attacked by a wide range of pathogen. Stalk rot caused by *Dickeya dadantii* (syn. *Erwinia chrysanthemi* (*Ech*) is one of the most destructive disease of sorghum crop in India(Samson *et al*., 2005). Recently, the increased stalk rot disease severity ranging from 7.50-46.85% in Tarai region of Uttarakhand (India) have been shown (Kharayat and Singh, 2013). The disease mainly affected sorghum stem showing water-soaked symptoms that later turned reddish dark brown color. The infected stem pith disintegrated and showed slimy soft-rot symptoms and eventually the whole plant wilted. *Dickeya dadantii* (syn. *Erwinia chrysanthemi* (*Ech*) is a motile, Gram-negative, non-sporing, straight rod with rounded ends, and occurs singly or in pairs: it varies from 0.8-3.2 x 0.5-0.8 µm (average 1.8 x 0.6 µm). There are 3-14, but more usually 8-11, peritrichous flagella (Burkholder, *et al*, 1953, Dickey, 1981) [7]. Pectic enzyme production and sensitivity to desiccation appear to be major factors in the biology of this bacterium. Plant infection by *E. chrysanthemi* and other soft rot erwinias causes various symptoms, both local and systemic, ranging from tissue maceration to wilts and blights (Pérombelon and Kelman, 1980; Kotoujansky, 1987; Expert, 1999; Yung *et al*., 2006) [11, 14, 8, 21]. *Dickeya dadantii* also produces two important siderophores (chrysobactin and achromobactin) that play important roles in its pathogenicity. The main objectives of these studies were conducted the pathogenicity test and established a suitable method for disease development through different artificial inoculation methods under glass house condition.Among these artificial methods, 'immersion of wounded roots in the bacterial cell suspension' method gave quicker symptom expression and highest lesion progression followed by 'stem injection inoculums', Leaf whorl, tooth pick and 'cotton wool inoculation' methods respectively. 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, 1972) [9]. Three parameters *viz.*, percent infected plants, length of water-soaked lesion progression and percent of disease severity (observed visually after splitting opening the infected stalks) by the *D.dadantii* were used.

Materials and Methods

Collection of diseased samples

Sample of naturally infected plants part of sorghum were collected with typical bacterial stalk rot symptoms will be collected from G.B.P.U.A & T, Pantnagar sorghum plot during kharif, 2016 and 2017. The infected aerial parts of the diseased samples was carefully placed in polythene bags, properly tagged and brought to the laboratory and then refrigerated at 4℃.

Isolation and purification of the pathogen

Infected plant portions of plants were cut into small pieces, surface sterilized with 0.1 percent sodium hypochloride (NaOCl) solution for two minutes and rinsed thoroughly thrice with sterile distilled water. The diseased bits were then transferred individually into a few drops of sterile water on a sterilized glass slide under aseptic conditions. The diseased bits were given a cut with sharp sterilized blade. The bits were left for one minute to allow bacterial ooze to come out in water. A charged needle with ooze was streaked onto crystal violet. 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 4^0C.Growth and maintenance of the bacterial culture were routinely grown on Luria-Bertani (LB) agar or LB broth at 25 - 28°C. Liquid cultures were shaken at 150-200 rpm in an orbital incubator (Gallenkamp). Cultures for routine use were stored at 4°C. For long-term storage, cultures were maintained in glycerol (15% final concentration) at -70°C.

Plant germination and growth for pathogenicity tests

Sorghum seeds were soaked in water and kept overnight at 30°C. Then the seeds were placed on moist filter paper at 30°C for 3/4 days. Germinated seeds were then sown in 4kg plastic pots. Pots with plants were kept in a growth cabinet maintained under 12 h photoperiod with a mean day: night temperature of 25: 20°C and a relative humidity of 80%. Twenty-one days old sorghum plants were used for the pathogenicity test.

Pathogenicity tests

To confirm the pathogenicity of isolate from Pantnagar, twenty-one days old plant inoculated with isolated bacterial culture. The bacterial culture was grown overnight at 25°C in 10 ml LB broth on an orbital shaker at 200 rpm. The resulting bacterial suspension was centrifuged at 3500 g in a bench top centrifuge for 5 min. The supernatant was discarded; bacterial pellet was resuspended in sterile 10 mM $MgCl_2$ solution and recentrifuged as described above. The washed pellet was again resuspended in 10 mM $MgCl_2$ solution and adjusted to a final concentration of bacterial suspension $2.5x10^8$ cfu/ml using at OD_{600} spectrophotometer and used immediately. Inoculum was tested on 21 days old susceptible sweet sorghum variety CSV19SS. Suitable controls were maintained using only distilled water in place of inoculum suspension. Leaf lesion length was measured from the cut surface at the tip to the distant-most position on the leaf that exhibited water-soaked symptoms that later turned reddish dark brown color.Data were taken from leaves of a plant at three-day intervals for up to 15 days after inoculation. Then pathogen was again reisolated from newly inoculated plants and compared with the original culture.

Inoculation techniques

Sorghum susceptible variety CSV19SS was grown in greenhouse condition (day/night temperature =32/25° C, 12 h photoperiod, and >90% RH). Plants of 21 days old were used to test the different inoculation methods. The bacterium suspension ($2.5x10^8$cfu/ml) was used as inoculum for spraying. The following six different inoculation methods were tried to find out most convenient and efficient method for disease production. These methods are described as under,

Immersion of wounded roots in bacterial cell suspension (**Bolick, 1960**)

Twenty-one days old plants grown were watered well 1hr before uprooting. The adhered soils to the roots were washed gently in tap water. Then again plants roots were washed in satirized water to avoid the undesirable soil-borne microbes or pathogens. The roots cut off about 3cm from the tip portion of main or primary root, and immediately immersed in the bacterial suspension for 10 min. Subsequently, the plants were transplanted into pots filled with sterilized, well watered soil in the glasshouse. The bacterial suspension was replaced with sterile 10m M MgCl2 in case of control.

Stem injection method

Bacterial suspension was inject-inoculated with a 21G 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) [3, 17, 15]. Control plants were inject-inoculated with sterilized water only.

Spraying the Inoculums with Hand Atomizer or Leaf-Whorl inoculation method (Klement, 1968) [13]

The pathogen usually enters the tissue through stomata, lenticels, hydathodes this method of inoculation is the most natural. The plants were kept in humid condition prior to inoculation to allow the stomata to open and to create high intercellular humidity in the tissues around the natural openings. The bacterial suspension was sprayed in leaf whorls (2ml/whorl) with hand atomizer. The inoculated plants were kept not to disturb the plants after inoculation so that maximum inoculum was retained in the leaf-whorls under high humid condition for 48 h by covering them with polyethylene bags and then left as such under natural condition. Plants sprayed with sterilized water served as control.

Tooth-pick method (Young (1943) [20]

Tooth picks were boiled thoroughly in water for two hours to remove resin, gum or any other toxic substances that might inhibit the growth of *D. dadantii*. After boiling, they were washed thoroughly in fresh tap water, and then tooth picks were dried in sun. About 10 tooth picks were placed in 100 ml flasks, in such a way that the pointed end of tooth picks faced away from the base and was autoclaved at 15 pounds psi (temperature 121^0C) for 20 minutes. *D. dadantii* was inoculated to 100 ml flasks containing sterilized LB, under aseptic condition, incubated at 28^0 Cand a rich suspension of bacterial cell was made within 7 days. This suspension was further poured into tooth pick containing flasks to cover lower $1/3^{rd}$ of the tooth picks under aseptic condition and incubated for seven days at 28^0 C, by the time, tooth picks were covered with the bacterial growth and were ready for inoculation. To confirm that toothpicks were colonized by *D. dadantii*, infested toothpicks were streaked onto NGM medium 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 tooth pick insertion. Tooth picks 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.

Midrib Injection Infiltration Method (Klement, 1963)
The method consists of injecting bacterial suspension into the intercellular spaces of leaves with a hypodermic needle. The hypodermic needle was inserted gently under the epidermis of the leaf. The opening face of the needle should be towards the leaf. Inoculations were made by injecting 0.1 ml of bacterial suspension in the leaf mesophyll so that tissue becomes water soaked.

Cotton wool method
Non-absorbent cotton wool was dipped in bacterial cell suspension and rubbed gently on the both surfaces of leaves to expose the maximum stomatal opening. Cotton wool dipped in sterilized water was used to rub the leaves of control plants.

Proving pathogenicity
Koch's postulates were followed to prove pathogenic nature of *D.dadantii*.

Statistical analysis
The experiment was done using ten plants in potted with each bacterial inoculation method in a completely randomized design. Each plant was considered as a replicate of each treatment. Due to repeated measurement of water-soaked lesion length and disease severity three analyses of variance (ANOVA) were carried out using the data recorded at 3, 6, 9, 12, 15-days after inoculation. Means and standard errors of means (SE) were also calculated.

Results
Pathogenicity of pathogen was confirmed on twenty one days susceptible sorghum plant variety CSV19SS in glass house by different inoculation method. Sorghum plants were inoculated by six methods resulted in the successful inoculation of *D. dadantii* suspension 2.5×10^8 cfu/ml and development of disease symptoms recorded up to 15 days in controlled environment conditions during kharif season in 2016. *D. dadantii* gave differential response with respect to method of inoculation. From the study it was exposed that the pathogen could effectively cause infection of the sorghum plant with varied incidence, intensity and some variation in symptom expression when inoculation was conducted by six inoculation techniques viz., Artificial inoculation by wounded roots dip and stem injection method of inoculation has been found effective in symptom development of bacterial stalk rot of sorghum (Hseu *et al*., 2008) [10]. The toothpick inoculation technique has been used to screen germplasm against sorghum and maize pathogens (Bramel-Cox and Claflin, 1989; Clements *et al*., 2003) [4, 6]. Inoculation via root wounding with a bacterial (*Ralstonia solanacearum*) water suspension in Kahilia ginger (*Hedychiumgardnerianum*), an invasive weed, has been shown by Anderson and Gardner (1999) [2]. The root tip cut & dip method of inoculation used in this investigation does stimulate most probable natural condition of infection to occur in many respects and is an efficient means of getting consistent and uniform infection of sorghum plants with *D. dadantii*. In natural conditions, roots get damaged when growing or penetrating the soil layer, to obtain required nutrients material. This damaged or wounded point can serve as mode of entry for the bacterium, as most do so. As this method gives uniform disease development in a quick time, it can also be used for determination of virulence and races of the pathogen in root rot type of diseases and plants in which transplanting is possible. *D. dadantti* required 3 days for production of small necrotic lesions. After 3 to 15 days of inoculation the spots were increased in size and turned dark brown, water-soaked which later developed into disintegration of pith tissues with foul smell. Wounded roots dip caused the almost rapid lesion formation and that data presented in table and figure that lesion progress was highest (22.4mm) after 15 days of inoculation. Disease progression over time depended on the inoculums dose and the inoculation method. Disease severity was rated using a numerical scale of the 0-5 index defined by Muhammad (1983), where 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 pith tissues at a single internode, premature of wilting 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. Liu *et al*. (1995), identified a disease severity index (DSI) for each replicate similar to the one used here. It was estimated along these linesDSI = Σs/(s max* n)*100; where s is the possible disease scores,max is the maximum disease score and n is the total number of plants examined in each replicate.

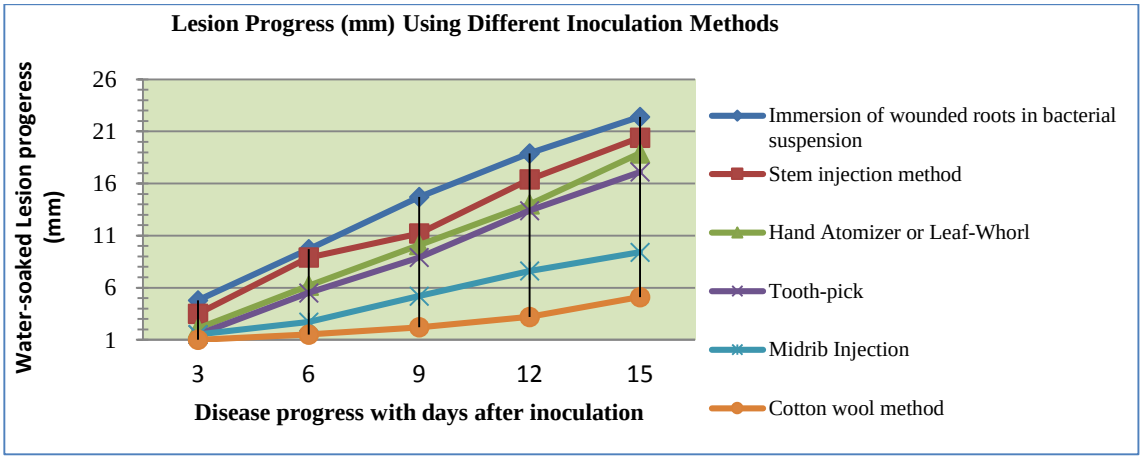

Fig 1: Lesion Progress (mm) Using Different Inoculation Methods

Table 1: Different methods of inoculation to find out a suitable method of producing bacterial Stalk rot disease in potted plants by using bacterial suspension (2.5x108 cfu/ml) (infected plant partrs or %)

Inoculation Methods	Potted Plants			
	Number of inoculated plants	Number of infected plants	infection percent	Disease severity index
Immersion of wounded roots in bacterialsuspension	10	10	100	89.75
Stem injection method	10	10	100	80.12
Hand Atomizer or Leaf-Whorl	10	9	90	74.45
Tooth-pick	10	9	90	70.08
Midrib Injection	10	8	80	61.80
Cotton wool method	10	7	70	54.76

Discussion

Results revealed that all methods produced stalk rot symptoms (Table 1). The stalk rot bacterium proved to be highly pathogenic, causing a rapid soft rot of parechymatous tissues on all inoculated plants (wounded roots dip and Stem injection).Water-soaked and discoloration of tissues (wet, glassy, yellow to brown steam lesions) around the inoculation court on sorghum plants was evident after three days of inoculation. After 10 days, wounded roots dip and stem injection caused significant difference and wounded roots dip inoculation was found to be better in all the cases irrespective of incubation period. However, lesion length varied depending upto extensive invasion of stem tissues were evident and plants toppled over in wounded roots dip inoculated method and plants developed rapid lesion after three days (Fig 1). After the development of infection plant disjointed apex appeared as a mushy foul smelling whip. Maximum disease severity (89.75%) was recorded with wounded roots dip methods followed by stem injection (80.12%), leaf whorl or automizer (74.45), tooth pick methods (70.08%), midrib injection (61.80) and Cotton wool method (54.76%). As the result was found consistent with glasshouse experiment, out of these six methods of inoculation wounded roots dip and stem injection methods can be considered as best and feasible. Inoculation with bacterial suspension in wounded roots dip andstem injection through hypodermic needle in punctures at the base of stalks of sorghum plants has been used (Saxena, *et al*., 1991) [19]. A rapid wounded roots dip technique recommended by Hartman and Kelman (1972) in corn for *Erwinia* soft rot was successfully used in sorghum plants also. However, the results of present findings were in disagreement from Allen *et al.*, (1981) [1]. He found that stem injection and leaf infiltration methods was reliable under greenhouse whereas, foliar sprays was most suitable for both greenhouse and fields. Klement (1963) reported injection infiltration as the best method for rapid test of pathogenicity with phytopathogenic *pseudomonas.* Further, Statistical analysis (Table1) showed significant difference between different inoculation methods.

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