Research Article

Optimization of Inoculation Technique of *Sporisorium scitamineum* for the Induction of Smut Disease in Sugarcane Propagative Material

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Abstract | Whip smut of sugarcane is one of the most important and destructive diseases of sugarcane, occurred in almost all sugarcane producing regions of the world. Its incidence could effectively be minimized by planting smut resistant cultivars. For evaluating a large number of propagative materials; standardized germplasm screening protocols as well as optimized inoculation techniques are required. The pathogen inoculation method should be less cumbersome, simple, and rapid. Here, we compared the effectiveness of six different inoculation methods, i.e., dipping, paste, wound+paste, soil infestation, spraying and injection methods for the establishment of smut disease. It appears that disease expression was significantly influenced by pathogen inoculation method. The infection caused by *S. scitamineum* and subsequent whip smut development has also been greatly varied with the inoculation method. Injection method followed by wound+paste method and dipping method produced maximum disease incidence, highest number of whips, more tillering and less germination. While, other inoculation techniques, including soil infestation and spraying method caused least infection that results in a comparatively low level of disease development. Diseased plants produced profuse tillers, but of no use as emerging tillers were very weak. Significant positive correlations were found among the disease incidence and other traits as well as between numbers of whips and tillers.

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Introduction

Whip smut of sugarcane caused by *Sporisorium scitamineum* (Syd.) (Piepenbr, 2002), formerly known as *Ustilago scitaminea* Syd., considered as the most important disease of sugarcane and occurred in almost all sugarcane producing regions of the world. It is basically a disease of meristematic tissues; pathogen only propagates in the young and actively growing plant tissues. Primary infection take place either by soil borne teliospores or by planting infected setts; while secondary infection occurs through airborne fungal spores infecting standing healthy crop (Santiago et al., 2012). It enters the healthy sugarcane plant through lateral buds, overcoming the barriers of bud scales. With the germination of an infected bud, the fungus also gets activated and grows along with the apical meristem and later on manifests itself in a very characteristic manner, i.e., by producing a typical black whip like smutty structure (Fontaniella et al.,

2002; Shamsul et al., 2012; Su et al., 2013).

The whip smut disease can effectively be managed by broad scale selection and planting of resistant cultivars as the most reliable, economic and effective control strategy (Schenck, 1998). Therefore, development of smut resistance is the focus of many sugarcane breeding programs worldwide (Chao et al., 1990). However, in sugarcane little work has been done regarding the development of standard germplasm screening protocols as well as optimization of pathogen inoculation techniques. An optimized inoculation method is the prerequisite for reliable and successful screening program. Different inoculation methods have been evaluated for the establishment of sugarcane whip smut disease (Srinivasan, 1969; Byther and Steiner, 1974; Lee-Lovick, 1978; Ferreria and Comstock, 1989; Wang et al., 2010; Huo et al., 2013; Shen et al., 2011, 2014; Olweny et al., 2008; Singh et al., 2014). For evaluating a large number of progenies, inoculation method should be simple, practical, rapid and reliable. The objective of this study was to assess the feasibility of inoculating sugarcane setts with smut and to evaluate the effectiveness of different inoculation techniques to accelerate the initial selection cycles of a breeding program for the development of smut resistant genotypes.

Materials and Methods

Experimental site and design

This research was conducted at National Sugar and Tropical Horticulture Crops Research Institute (NSTHRI) Makli, Thatta, Sindh, Pakistan. The variety CP29-120 was selected for this experiment, which proved highly susceptible to the sugarcane smut pathogen in cultivar screening studies. For each inoculation method, 42 single budded setts of this cultivar were collected from disease free crop. After inoculation, these single budded setts were sown in the plastic bags of 25×35 cm; containing 7 kg sterilized sandy loam soil. The trial was laid out in a Randomized Complete Block Design (RCBD) with three replications. Each replication comprised of 7 bags; while, 2 setts were sown in each bag.

Teliospore collection, preservation and inoculum preparation

Fresh smut whips were collected from smut affected plants grown in experimental field of NSTHRI. After shade drying, the teliospores were gently scraped and thoroughly sieved, using 53 µm mesh. The sieved teliospores were sealed in cellophane bags and stored in the refrigerator at 10°C. Viability of inoculum was confirmed on water agar and those shown viability of >70% were taken for preparation of inoculum suspension. Four grams of spores were added in 1 litre of distilled water along with a few drops of Tween® 20 for homogenous spore suspension. Finally, the inoculum density was adjusted to 4×10^6 spores/ml with the help of haemocytometer (Nasr, 1977).

Inoculation techniques

Six different inoculation methods, i.e., dipping, paste, wound+paste, soil infestation, spraying and injection methods were evaluated. The setts dipped in distilled sterilized water served as control. The details of each method are as under:

Dipping method: The apparently healthy **s**ingle budded setts of cv. CP29-120 were dipped in *Sporisorium scitamineum* spore suspension (4×10^6 spores/ml) for 30 minutes and incubated in polythene bags for 24 hours to facilitate pathogen penetration and planted in plastic bags at 2 setts/bag.

Paste inoculation: The teliospores paste has been prepared by adding few drops of sterilized water in 4 g of *Sporisorium scitamineum* spores. With the help of drawing brush, it was thoroughly applied on the surface of the buds. After pasting, the inoculated buds were kept in polythene bags for 24 hrs before being planted.

Wound paste inoculation: Prior to the inoculation, the setts were wounded with the help of sterilized surgical pin. Four pinholes were made around the periphery of each bud and then pathogen paste was applied as described above. After inoculation, these buds were placed in polythene bags for 24 hrs before planting in the plastic bags.

Soil infestation method: Before sowing of buds, 4 gram teliospores were thoroughly mixed in sterilized soil and then filled in plastic bags. Two single budded setts were planted in each plastic bags and watering was practiced as usual.

Spraying: The setts were inoculated by spraying the already prepared pathogen inoculum suspension ($4 \times 10_6$spores/ml) with the help of atomizer. After spraying, the inoculated setts were kept in polythene bags for 24 hrs before planting in the plastic bags.

Injection method: One ml of already prepared teliospores suspension ($4 \times 10_6$spores/ml) was injected in the cane buds with the help of a hypodermic syringe. After injecting the inoculums, buds were kept in polythene bags for 24 hours and then planted in the plastic bag containing 7 kg sterilized sandy loam soil.

Data collection

Data on sett germination, tillering and disease development was recorded from sowing to six months of plantation. Germination of buds in each treatment was recorded till 30 days of sowing. Observations on smut incidence were recorded fortnightly till six months of sowing by counting emerging whips in each treatment. After each observation, the whips were removed. After six months of sowing, the cumulative disease incidence in each of the inoculation method was calculated with the help of following formula:

$$\text{Disease incidence}(\%) = \frac{\text{Number of diseased stools in each treatment}}{\text{Total number of germinated stools in each treatment}} \times 100$$

Results and Discussion

Effect of inoculation method on bud germination

Inoculation methods of *Sporisorium scitamineum* greatly influenced on the germination of sugarcane buds. The wound+paste method followed by injection method, remarkably reduced the germination of buds. Buds treated with these two methods showed 54.76 and 61.9% germination, respectively. The dipping method has also moderately affected the germination. While, other methods have influenced less on the bud germination. After control (un-inoculated), the highest germination occurred in buds inoculated by soil infestation method (78.57%), followed by paste method (73.81%) and spraying method (76.19%) (Figure1a).

Disease incidence

The infection caused by *S. scitamineum* and subsequent whip smut development has been greatly varied with the inoculation method. Some methods produced a very high level of disease, while others produced less. Significantly, maximum disease incidence of 57.41% was observed in injection method, followed by wound+paste method which produced 51.05% incidence of whip smut. Soil infestation and spraying of spore suspension appeared least effective, caused lowest disease development in inoculated buds. Moderate disease incidence was recorded in plants inoculated with dipping and paste methods (Figure 1b).

Tillering

The successful infection of *S. scitamineum* in planting material enhanced the profuse tillering. Accordingly, more increased tillering was observed in treatments which has high levels of disease. Significantly, maximum number of tillers was recorded in injection method (7.84) followed by wound+paste (7.56) and dipping methods (7.30). While, un-inoculated as well as plants inoculated by soil infestation method and spraying method produced a minimum number of tillers as compared to the rest of the others (Figure 1c).

Number of whips

The average number of whip structures developed in each treatment was also varying with the pathogen inoculation method. Setts inoculation by injecting the teliospores suspension produced significantly higher numbers of whip i.e. 14/stool. The 2nd highest numbers of whips were developed in plants inoculated with wound+paste and dipping method. On the other hand, a significant minimum number of whips appeared in soil infestation method (Figure 1d).

Regression analysis of disease incidence with tillers, number of whips and germination as well as between numbers of whips and tillers were calculated using the linear model in Microsoft Excel. A strong uphill positive correlation was found between the compared parameters. In case of the relationship between disease incidence and tillers coefficient of determination (R^2) was 0.66, between disease incidence and number of whips was 0.94, disease incidence and germination % was 0.86 while between numbers of whips and tiller was 0.68 (Figure 2).

In plant disease research, scientists have successfully developed protocols for screening of germplasm against a wide range of plant pathogens. In recent years, a number of clone/smut evaluations has been undertaken around the world with varying results. Factors responsible for the varying results include the presence of different pathogenic races, variation in clones due to location, and different inoculation techniques. Over the years the inoculation methods/techniques for various diseases were developed and standardized. Like cereal rusts and smuts, researchers typically use different inoculation methods or assays

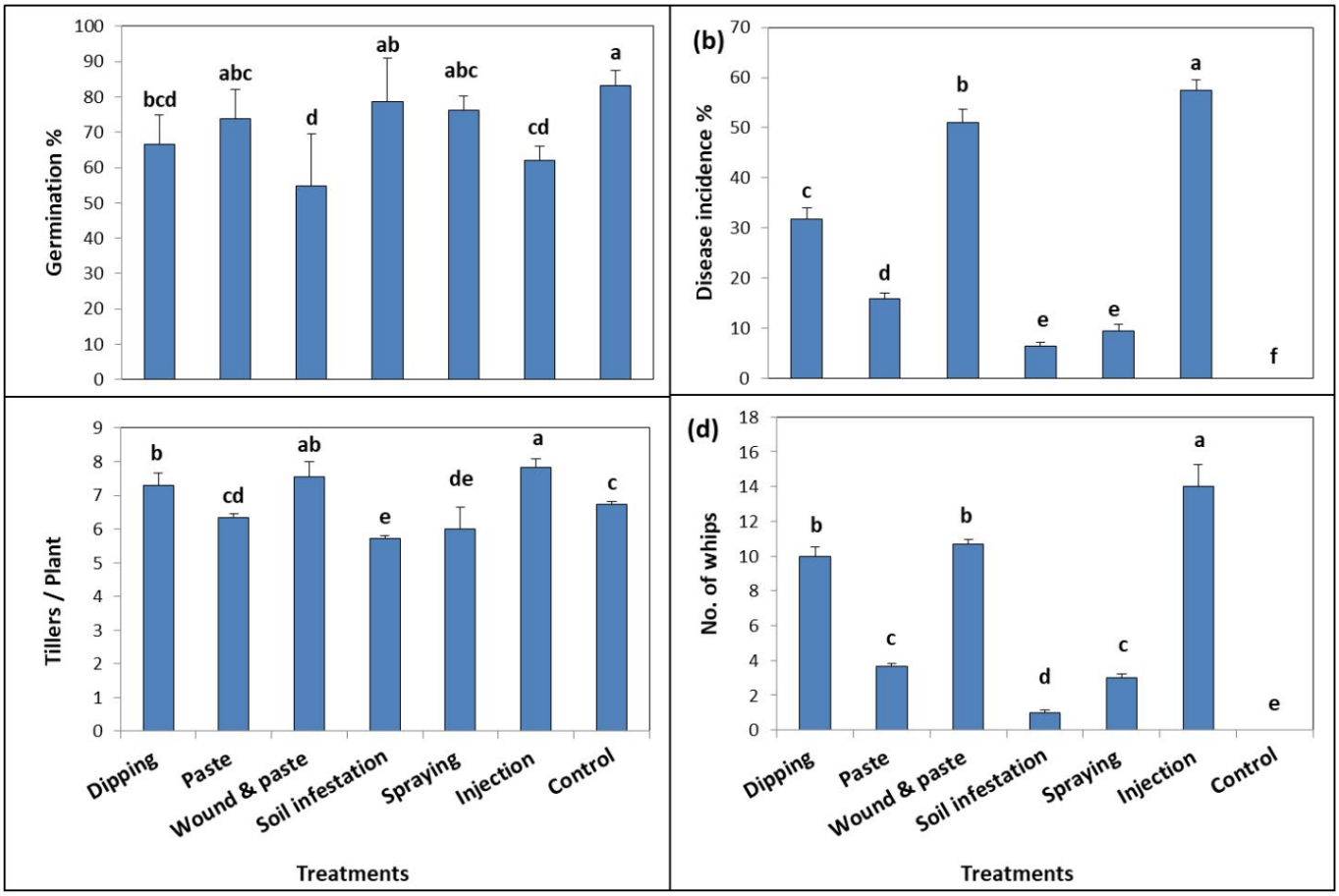

Figure 1: *Comparative response of sugarcane single budded setts inoculated with whip smut pathogen Sporisorium scitamineum by different methods; these were evaluated for (a) germination, (b) disease incidence (c) tillering and (d) number of whips. Each treatment replicated three times, i.e., consists of seven bags with a total number of 42 single budded setts. The error bars represent the standard deviation (SD). Bars labeled with the different letters indicating that the mean values are statistically different ($p < 0.05$, Tukeys test).*

(Olweny et al., 2008). Artificial inoculation can be achieved by various inoculation techniques (Ilyas et al., 1992). Selveraj (1980) and Kutama et al. (2011) reported six different inoculation methods for head smut and two for grain and loose smuts. However, in some cases the standard inoculation methods are still not available, including whip smut of sugarcane. A successful program in sugarcane breeding for resistance to smut disease, caused by *S. scitamineum*, requires an effective inoculation method, that is practical, rapid and reliable. Usually, for large scale inoculation of sugarcane planting material, setts (seed pieces with 1-3 nodal buds) were dipped in spore suspension before sowing or by painting the buds with the spore suspension and then planted in the field (Byther and Steiner, 1974; Lee-Lovick, 1978; Ferreria et al., 1989; Wang et al., 2010; Dalvi et al., 2012; Huo et al., 2013; Shen et al., 2011, 2014). The use of teliospore suspension has already been demonstrated as a large amount of inoculum for detection of sugarcane resistance to *S. scitamineum* disease, but due to its complexity like collecting teliospores in the field, unstable field results due to lack of uniform inoculum, impossibility in identifying the physiological race, etc. made it unappealing to investigators. Moreover, the inoculation process must result in sufficiently high levels of disease to discriminate between susceptible and resistant genotypes. Therefore, we tried 6 different inoculation methods to infect sugarcane planting materials (single budded setts) with teliospores of *S. scitamineum*. We found that for maximum disease development injection method and wound+ paste methods were more helpful than others. In both methods, the casual pathogens easily reach within the host tissues, thereby speed up the pathogenesis process that results in increased symptom expression. Other workers have also found that the efficient and swift pathogen penetration in the host cell resulted in higher levels of whip smut development in inoculated germplasm. Some studies revealed that injection method was the most suitable technique for whip smut development (Singh et al., 2014; Waller, 1970); while, others found a pin prick method more effective (Gao et al., 2013; Marchelo et al., 2008).

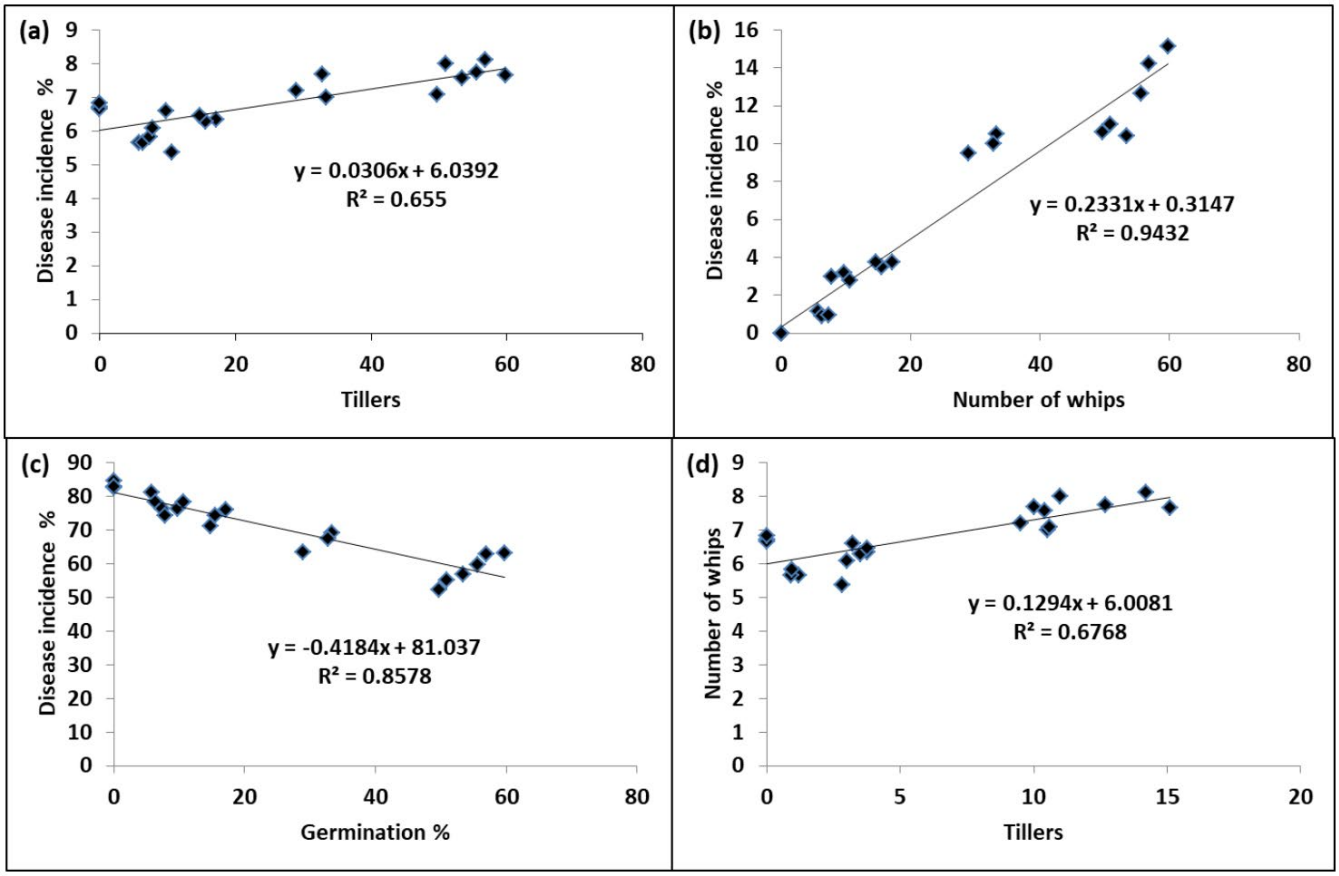

Figure 2: *Correlation between plant growth and disease development. Calculated using the linear model in Microsoft excel using 21 replications of seven different methods of inoculation for sugarcane by Sporisorium scitamineum. Disease incidence with tillers (a), disease incidence with number of whips (b), disease incidence with germination (c) and between numbers of whips and tillers (d).*

Conclusions and Recommendations

We concluded that, injection method and wound+paste method are the two most reliable and efficient techniques, which produced maximum smut development in inoculating material. It happens because they avoid any physical hindrance for reaching the pathogen inoculum within the host tissues. The contact between *S. scitamineum* teliospores and host cells results in the successful infection and whip smut expression, if the cultivar is susceptible.

Author's Contributions

Muhammad Aslam Rajput: Execution of the experiment and data collection.
Imtiaz Ahmed Khan: Planning, assembly and possession of the raw data.
Rehana Naz Syed: Analysis and interpretation of data.
Abdul Mubeen Lodhi: Overall supervision, critical revision and final approval MS for publication.

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