Plant Health Initiative

Development of a standardized method for rice neck blast inoculation

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Abstract

Rice blast disease caused by *Magnaporthe oryzae* B.C. Couch causes significant yield losses in rice production globally. Despite its economic importance, the epidemiology of neck blast is not well understood which could be partly attributed to the absence of a reliable and standardized method for inoculating the neck with the pathogen. This study was conducted to develop a reliable and replicable method for inoculating the rice neck with the blast pathogen. The method was developed based on ten replicated experiments conducted in a greenhouse at the International Rice Research Institute (IRRI) 2018 to 2020. The results showed that the injecting the spore suspension into the neck is the most effective delivery method, followed by pricking the neck and then wrapping it with cotton soaked in spore suspension, brushing the neck with spore suspension, wrapping the non-pricked neck with cotton soaked in spore suspension, and spraying. The injection method and spore concentration of 1 x 10^5 spores/ml resulted in higher neck blast incidence, longer lesion and relative lesion length, and shorter incubation period compared to the other treatments. The best inoculation method is injection on Co39 or NSIC Rc216 variety using the following components: downward direction of injection, 1 x 105 spores/ml spore concentration, 0.25 mm x 8 mm needle size, 0.3 ml volume, and inoculation at heading stage. Additional experiments will be conducted at IRRI to improve the inoculation method developed in this research. The effect of growth stage on the efficacy of the injection method will be evaluated to optimize the timing of its application. The growth stages to be considered are heading, flowering and milk because infection at these stages affect neck blast incidence under natural conditions.

Keywords: neck blast, injection method, inoculum delivery system

Introduction

Blast is caused by *Magnaporthe oryzae* B.C. Couch (anamorph *Pyricularia oryzae*), formerly known as *Magnaporthe grisea* (Hebert) Barr (Galhano and Talbot, 2011). It is considered as one of the most economically important diseases of rice, causing annual yield losses ranging from 10 to 30% and at least 70% in severe cases (Talbot, 2003; Skamnioti and Gurr, 2009; Nalley et al., 2016). Controlling blast increases the cost of pesticide application during severe epidemics, and opportunity cost in maintenance breeding (Nalley et al., 2016). Severe neck blast negatively affects both the quantity and quality of the grains (Shim et al., 2003; Koutroubas et al., 2009; Khan et al., 2014).

The most common forms of blast are leaf blast and neck blast. Although the pathogen targets all aboveground parts of the rice plant throughout its life cycle, it usually infects the leaves and the neck. During the vegetative stage, the pathogen starts to infect the leaves causing leaf blast, which is characterized by elliptical lesions. These lesions could enlarge and coalesce causing premature senescence due to a reduction of the photosynthetic rate of leaves (Bastiaans et al., 1994; Wang et al., 2014) and an enhanced rate of respiration (Bastiaans et al., 1994), and eventually results in death of the leaves.

During the reproductive and ripening stages, the pathogen infects the neck (the uppermost node located just below the panicle), resulting in neck blast or neck rot (Ou, 1985). A lesion on the neck causes necrosis and premature senescence, disrupts translocation of assimilates from the leaves and stem and water transport from the roots to the developing grains. Neck blast is considered as the most destructive form of blast. Grains are not produced if the pathogen infects the neck before the milk stage. If the infection occurs later, neck blast leads to partially filled spikelets and grains of poor quality. Neck blast causes the tiller to break at the infected neck, resulting in the loss of the entire panicle.

The epidemiology of neck blast is considerably less understood than that of leaf blast, despite its economic importance. This can be attributed to the prevailing assumption that weather effects and other risk factors are similar for both leaf blast and neck blast and that leaf blast progresses into neck blast if left uncontrolled. Another important reason is the absence of a reliable and standardized method for inoculating the neck with the pathogen. Artificial inoculation is crucial for achieving the desired level and timing of disease incidence or severity and ensuring a sufficient and uniform distribution of disease symptoms in plants (Sørensen et al., 2016). It could help minimize experimental variability and effects of confounding factors, which can be difficult to control when working with naturally infected plants. A reliable inoculation method is more crucial for studying neck blast epidemics than for leaf blast, as neck blast occurs only during a specific and shorter period of the rice cropping season.

Several methods to artificially deliver the inoculum, usually in the form of spore suspension, to the neck have been developed. These include the brushing the neck with the spore suspension (Vingnanakulasingam, 1991; Sun et al., 1992; Ghatak et al., 2013); placing bits of leaves from *Paspalum dilatatum* (Singh et al., 2021) or *Brachiaria mutica* (Bhaskar et al., 2017) that contain spores on the neck; wrapping or tying the neck with cotton (Vingnanakulasingam, 1991; Roumen, 1992; Sun et al., 1992; Yang, 2013; Ghatak et al., 2013; Bhaskar et al., 2017), string (Vingnanakulasingam, 1991) or paper twine (Koga and Tsukamoto, 2011) soaked in spore suspension; injecting the spore suspension into the neck (Vingnanakulasingam, 1991; Sun et al., 1992; Vaishali et al., 2007; Ghatak et al., 2013; Yang, 2013; Bhaskar et al., 2017) or entire leaf sheath (Koga et al. 2004); placing plastic rings filled with conidial suspension around the neck (Koga, 1994); spraying the conidial suspension onto the neck (Vingnanakulasingam, 1991, Sun et al., 1992; Koga and Tsukamoto, 2011; Yang, 2013; Ghatak et al., 2013, Chi and Park, 2015; Singh et al., 2021); and placing slices of culture medium with the sporulating pathogen and spores directly onto the neck (Korinsak et al., 2023). Despite all various methods, a standard method for neck blast inoculation has yet to be established.

This research study was conducted to develop a reliable and replicable method for inoculating the rice neck with the blast pathogen.

Materials and Methods

General methodology

Ten experiments were conducted in BGO3, a greenhouse located at the New Greenhouse of the International Rice Research Institute (IRRI), Los Baños, Laguna from January 2018 to January 2020 to develop a method for inoculating the neck of experimental plants with spore suspension of the *M. oryzae*. The results of these experiments served as the basis for developing an inoculation method for analyzing the effect of weather parameters and other risk factors on neck blast epidemiology under controlled conditions.

Establishment of experimental plants

Rice seeds of the test varieties were soaked in continuously flowing water for 24 h and then air-dried for another 24 h. Germinated seeds were then grown in trays with sterilized soil and then transplanted in pots after one week. Each pot was filled with 1.75 kg sterilized soil, and 0.83 g ammonium sulfate (21% nitrogen) pot one day before seedlings were transplanted. In each pot, five 7-day-old seedlings were transplanted. Plants were maintained in the greenhouse until they reached the preferred growth stages of an experiment (Figure 1). Co39 was grown as the standard test variety because of its high susceptibility to blast.

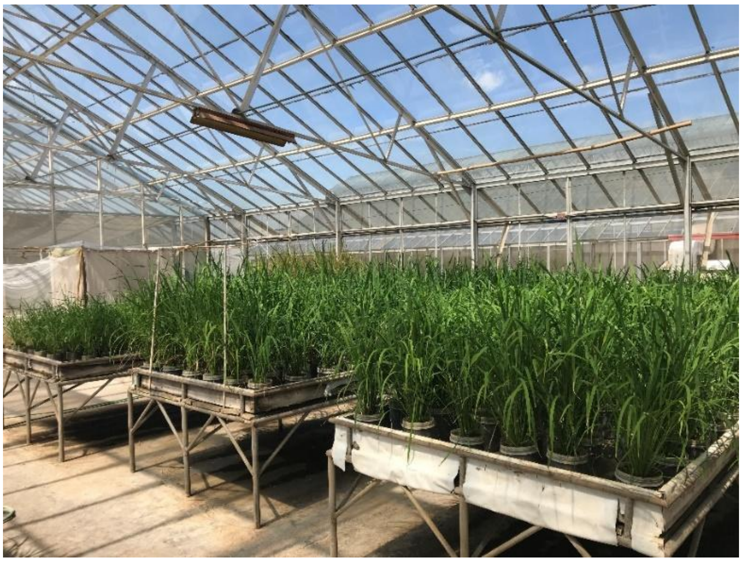

Figure 1. Experimental plants were maintained in the New Greenhouse of the International Rice Research Institute until the required growth stage of an experiment.

Preparation of inoculum

BNIII (Blast Nursery III) isolate of *M. oryzae,* which is virulent to neck blast, was used in all experiments. The isolate was obtained from the collection maintained at the Plant

Pathology Laboratory of IRRI. It was isolated from an infected rice plant in the IRRI Blast Nursery. To culture the isolate, 10 to 15 ml of sterilized prune agar medium was used. This medium was prepared by boiling three pieces of prunes (*Prunus domestica* L.) in distilled water for 30 minutes. After boiling, the mashed prunes were filtered through a nylon mesh to extract prune juice. The extract was then mixed with 21 g of agar and boiled for an additional 20 minutes. Subsequently, 5 g of lactose monohydrate and 1 g of yeast extract were added and stirred well. The volume of the mixture was adjusted to 1 liter by adding distilled water, and NaOH solution (8 g in 200 ml distilled water) was incorporated to set the pH at 6. The prepared medium was autoclaved for 20 minutes at 15 psi (121°C).

Before pouring the melted sterilized prune agar medium into Petri dishes, 40 mg/L of Streptomycin was added to prevent bacterial contamination. A 5-mm mycelial plug of the fungus was placed on the prune agar medium and incubated at 28°C for five to seven days. After this incubation period, the growing mycelia were gently scraped with a sterilized glass slide and exposed to alternating cycles of light and dark (12 hours light/12 hours dark) under fluorescent light for three to four days to induce sporulation (Figure 2).

To harvest the spores, 10 to 15 ml of sterile distilled water was poured onto each Petri dish. The resulting solution was then transferred to a beaker through sterile Miracloth to filter out mycelial fragments. The spore suspension was standardized to 5 x 10^5 spores/ml using a hemocytometer and mixed with 0.02% Tween 20 (Polyoxyethylene 20 sorbitan monolaurate; Sigma Aldrich) to prevent clumping in the Petri dish.

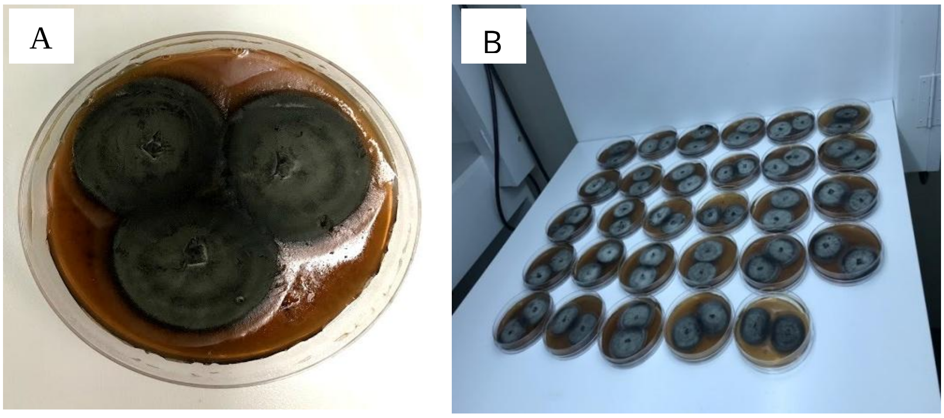

Figure 2. A. Plate culture of BNIII isolate of *Magnaporthe oryzae*, B. The plate cultures of BNIII isolates exposed under fluorescent light at an incubator room to induce sporulation.

Inoculation of test plants with the blast pathogen

The basic unit in all experiments consists of two hills separately grown in pots, measuring 16 cm height, 11 cm diameter. Inoculation methods were tested on a sample of five tillers of a potted plant. The concentration and method of spore suspension was applied according to the experimental treatments. In all experiments, the spore suspension was kept in an ice box before the application to maintain the viability and inhibit the germination of spores and prevent evaporation. For the spraying method, the inoculum was applied until runoff occurred using a sprayer that produces a fine mist. The sprayer was aimed at a distance of 10 to 15 cm from the neck. For the injection method, specific concentrations of spore suspension were injected into the neck using a syringe equipped with a 0.50 x 16-mm hypodermic needle (Terumo® syringe, 25G x 5/8"). The syringe was inserted into the neck up to the slit of the needle at a slight angle of 15 to 20°.

Maintenance of inoculated plants

All the inoculated plants were placed in a room with a temperature of 25 to 26°C. After three days, the plants were transferred into a cage (2.0 m x 3.0 m x 2.2 m) covered with a jute sack until neck blast symptoms were observed (Figure 3). The jute sacks were sprayed with water three to four times a day to achieve high humidity and maintain the temperature at 27 to 28°C. Plants were also sprayed until runoff with water using a sprayer with fine mist (water flow 550 ml/min) to facilitate disease development without damaging the panicles.

A data logger with weather sensors (HOBO, Onset computer cooperation) and a manual hygrometer were placed inside the jute chamber to monitor air temperature and relative humidity inside the jute cages.

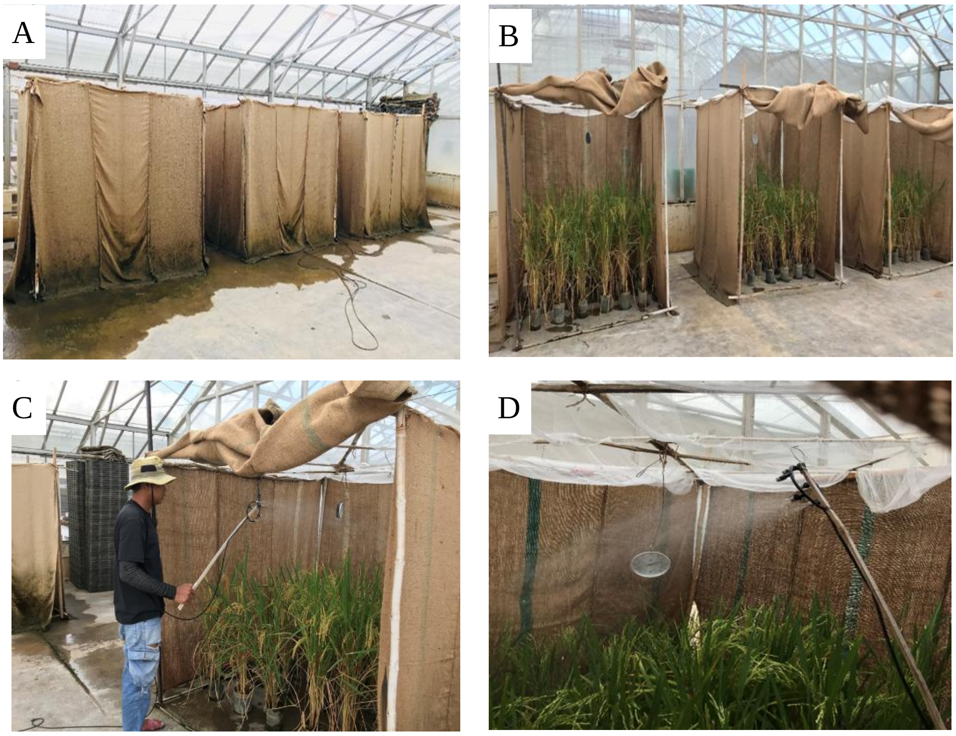

Figure 3. Inoculated plants were maintained in cages covered with Jute sack in the greenhouse. (A) Cages covered with Jute sack, (B) arrangement of the experimental plants inside the cage, and (C and D) spraying of test plants with fine mist of water to enhance disease development.

Experimental design and treatments

Experiment 1. Effect of variety, inoculation method, and plant growth stageon neck blast incidence

Experiment 1 was conducted from January to March 2018. The experiment was laid out in a split-split plot design (Gomez and Gomez, 1984) with three replications. The main plots were three varieties, namely, IR50, Co39, and NSIC Rc216 (National Seed Industry Council Rc-216; Tubigan 17). The subplots were two growth stages, (late booting and heading) and the sub-sub plots were delivery two methods (spraying and injection). Establishment of test plants and injection of spore suspension and data collection and analysis were conducted as described in the general methodology.

Experiment 2. Effect of variety and inoculum concentration on neck blast incidence

Experiment 2 was conducted from February to April 2018. Treatments were arranged in a split plot design with three replications. The main plot factor was variety (Co39, IRBL1, NSIC Rc216), and the subplot factor was the amount of the inoculum (2.5 x 10^4 spore/ml and control (0 spore/ml)). The inoculum was injected into the neck using a syringe as described in the general methodology.

Experiment 3. Effect of variety, and inoculum concentration at heading stage on neck blast incidence and incubation period

Experiment 3 was conducted from April to August 2018. Treatments were arranged following a split plot design with three replications. The main plot factor was variety (Co39 and NSIC Rc216) and the subplot factor was the concentration of the inoculum (0, 5 x 10^4, 1 x 10^5, and 2 x 10^5 spores/ml). At heading stage, the spore suspension was injected into the neck using a syringe, as described in the general methodology.

Experiment 4. Analysis of the effect of inoculum concentration and syringe needle size used for injection of inoculum on neck blast incidence, lesion length, and incubation period

Experiment 4 was conducted from September to December 2018. It was laid out in split plot design and replicated three times. The main plots were three concentrations of inoculum (0, 1 x 10^4, 1.5 x 10^4, and 2 x 10^4 spores/ml), and the subplots were three sizes of hypodermic syringe needle (0.25 x 8 mm - 31 G, insulin syringe and 0.50 x 16 mm – 25G x 5/8" disposable syringe needle, Figure 4). Inoculum was injected into the neck, as described in the general methodology. Co39 was used in this experiment.

Figure 4. The syringes used in Experiment 4. (A) 0.25 x 8 mm - 31 G, hypodermic syringe. (B) 0.50 x 16 mm – 25G x 5/8” (Terumo® syringe).

Experiment 5. Effect of inoculum concentration and direction of syringe during injection on neck blast incidence and incubation period

Experiment 5 was conducted from April to June 2019. It was arranged in split plot design with the concentration of inoculum (0, 1 x 10^4, 2.5 x 10^4, 5 x 10^4, and 5 x 10^5 spore/ml) as main plot and two positions of the syringe during injection (downward and upward) as subplot. The experiment was replicated three times.

The neck of five tillers in each pot was injected with 0.3 ml volume of spore suspension using a hypodermic syringe (BD Ultrafine-II^{tm}, 31G, 0.25 x 8 mm). For the upward and downward positions, the needle was angled 15 to 20° upward or downward, respectively, relative to the horizontal plane (Figure 5).

Figure 5. The upward and downward positions of the syringe during infection of spore suspension.

Experiment 6. Effect of inoculum concentration, growth stage, and delivery volume during injection on neck blast incidence and incubation period

Experiment 6 was conducted from August to October 2019. It was laid out split-split plot design with three replications. The main plot factor was the concentration of inoculum (0, 2.5 x 10^4, and 5 x 10^4 spore/ml), the subplot factor was growth stage (booting and the heading stages), and the subsubplot factor was volume of the inoculum during delivery, (0.3 and 0.5 ml). Co39 was used in this variety.

Experiment 7. Effect of inoculum concentration and heading percentage during injection on neck blast incidence and incubation period

Experiment 7 was conducted from June to August 2019. Experimental treatments were arranged in a split plot design with three replications. The main plot factor was the concentration of inoculum (0, 1 x 10^4, 5 x 10^4 spore/ml) and the subplot factor was percentage of panicles at heading stage (25% and 50% heading). NSIC Rc216 was the variety used in the experiment. Five tillers of each pot were injected according to required concentration of inoculum at 25% or 50% heading stage. The inoculum was injected into the neck as described in the general methodology.

Experiment 8. Effect of inoculation delivery methods on neck blast incidence and lesion length

Experiment 8 was conducted from August to October 2019. Treatments were arranged in a randomized complete block design with three replications. The treatments consisted of five inoculum delivery methods: (1) spraying the neck with spore suspension, (2) brushing the spore suspension onto the neck, (3) wrapping the neck with cotton was soaked with the spore suspension, (4) pricking the neck and then wrapping it with cotton soaked in spore suspension, and (5) injection of the spore suspension into the neck as described in the general methodology.

The spore concentration in all inoculation methods was 2.5 x 10^4 spores/ml.. Five tillers from each pot were inoculated according to the specified methods. In the first method, a handheld sprayer was used to apply the spore suspension onto the neck until it began to run off. In the second method, a no. 4 paintbrush was used to apply the spore suspension to the neck. In the third method, 0.3 ml spore suspension was applied to a 15- x 17-cm single layer of cotton using a 5-ml disposable syringe needle (Terumo® syringe, 25G x 5/8" 0.50 x 16mm). The cotton was then wrapped around the neck. The fourth method was the same as that of the third method, except that the neck was gently punctured using a syringe needle (BD Ultrafine-II^{tm} short needle 0.25 mm (31G) x 8 mm) before cotton soaked in spore suspension was wrapped around the neck. In the third and fourth methods, plastic was wrapped around the neck along with cotton to enclose it and retain moisture to create a favorable condition for infection. In the fifth method, 0.3 ml of spore suspension was injected into the neck using a 0.5 ml insulin syringe needle (BD Ultrafine-II^{tm} short needle 0.25 mm (31G) x 8 mm). During injection, the needle was angled downward at 15 to 20°.

Experiment 9. Effect of variety, inoculum concentration, and inoculation method on neck blast incidence and lesion length

Experiment 9 was conducted from October to November 2019. Treatments were arranged in a split-split plot design with three replications. The main plot factor was variety (Co39, and NSIC Rc216), the subplot factor was the concentration of inoculum (0, 2.5 x 10^4, and 1 x 10^5 spores/ml), and the sub-subplot was inoculum delivery method (cotton with pricking of the neck and injection). Inoculation was done at flowering stage.

In the cotton with pricking method, the neck of five tillers of every potted plant was slightly punctured using a 0.5 ml hypodermic insulin syringe needle (BD Ultrafine-II^{tm} short needle 0.25 mm (31G) x 8 mm). Approximately 0.3 ml spore suspension with different concentrations was dripped into a 15-x 7-cm single-layer cotton using a 5-ml disposable syringe needle (Terumo® syringe, 25G x 5/8", 0.50 x 16mm) and then wrapped around the rice neck. A sheet of Klinpak Cling Wrap® (approximately 5 cm in length) was used to enclose the cotton to maintain moisture. The injection method was performed by injecting the spore suspension into the neck. The syringe needle containing the spore suspension was injected into the neck with the needle angled downward at 15 to 20°.

Experiment 10. Effect of variety, inoculum concentration, and inoculation method on neck blast incidence, incubation period, lesion length, and relative lesion length

The last experiment was conducted from November 2019 to January 2020. The experiment was conducted to evaluate the effects of the method, developed based on results from previous experiments, on various disease variables and to determine whether further optimization of the method was necessary. Treatments were laid out in split-split plot design with three replications. The main plot factor was variety (Co39 and

NSIC Rc216). The subplot factor was the concentration of the spore suspension (0, 2.5 x 10^4, and 1 x 10^5 spores/ml)., and the sub-sub plot factor was delivery method (injection, cotton without pricking, and cotton with pricking of the neck using the needle). The inoculation methods were described previously. Inoculation was performed at flowering stage.

Inoculation of the neck with *Magnaporthe oryzae* isolate BNIII

Every ten tillers from four hills of Co39 and NSIC Rc216 rice varieties from two pots were inoculated with 0, 2.5 x 10^4, and 1 x 10^5 spores/ml of the spore suspension using the injection method, and the cotton with and without neck pricking methods at flowering stage (Figure 6). The injection method involved administering 0.3 ml of spore suspension into the neck using a 0.5 ml hypodermic insulin syringe (BD Ultrafine-II^{tm}, 31G, 0.25 mm x 8 mm). The syringe needle, containing the spore suspension, was inserted at a slight angle of 15-20° into the rice neck, with the needle positioned downward. For the cotton with and without neck pricking methods, 0.3 ml spore suspension was dripped into 15 x 7 cm single layer cotton using a 5 ml disposable syringe needle (Terumo® syringe, 25G x 5/8", 0.50 x 16mm), before wrapping around the rice neck. Additionally in the cotton with neck pricking method, before applying the cotton, the rice neck was slightly punctured first using a 0.5 ml hypodermic syringe needle (BD Ultrafine-II^{tm}, 31G, 0.25 mm x 8 mm) to make a wound without damaging the rice neck. Plastic wrapping (approximately 5 cm in length) was used to enclose the cotton segment to maintain moisture and provide a favorable condition for pathogen infection. Cotton was removed from the neck after three days, to allow the plant growth and development.

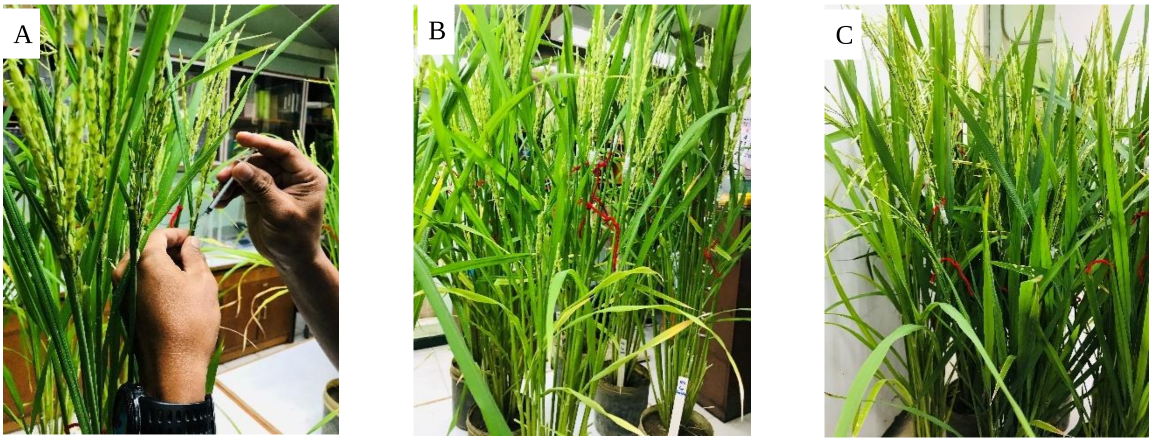

Figure 6. Application of the spore suspension to the neck on different rice varieties using (A) injection, (B) cotton without pricking the neck and (C) cotton with pricking of the neck.

Neck blast incidence and incubation periods were measured after inoculation as described in the general methodology. Lesion length was measured as the length of the necrotic area or lesion on the infected neck using a ruler. Relative lesion length (RLL) was calculated as percentage the length of the neck blast lesion to the total length of the aboveground portion of the tiller.

Data collection and analysis

Neck blast incidence was assessed in all experiments as the percentage of panicles in a pot with typical neck blast symptoms. Incubation period was assessed as time interval, in number of days, between inoculation and the appearance of the first visible symptoms of neck blast.

Data on disease variables were analyzed using ANOVA, followed by post hoc tests. Least significant difference (LSD) test was used for pairwise mean comparisons. Shapiro-Wilk Test was used to determine whether the collected data or residuals follow a normal distribution and ensure that data is appropriate for parametric analysis, Bartlett's test was used to determine the equality of variances across groups or treatment means (homoscedasticity). Data that violated the assumptions of normality or equal variances were transformed using square root transformation. All statistical tests were conducted at a significance level of $p \leq 0.05$. Data were analyzed using Statistical Tool for Agricultural Research (STAR) v 2.0.1 (IRRI, 2013).

Summary of experiments

Table 1 summarizes the description of all experiments. Figure 7 illustrates the flow diagram for the development of the neck blast inoculation method based on the different experiments.

Table 1. Summary of experimental treatments for the development of the method for neck blast inoculation.

Variety	Inoculum concentration (spore/ml)	Delivery system: Delivery method	Injection method: Vol. (ml)	Injection method: Needle size	Injection method: Direction of syringe	3. Plant growth stage	Experimental design
Experiment 1. Effect of variety, inoculation method, and plant growth stage on neck blast incidence							
1.. IR50 2. Co39 3. NSIC Rc216	5 x 10^5	1). Spraying 2). Injection	0.5	25G x 5/8” 0.50 mm x 16 mm	Upward	1. Late booting 2. Heading	Split-split plot with 3 replications Main plot: Variety Sub plot: Plant growth stage Sub-sub plot: Inoculation method
Experiment 2. Effect of variety and inoculum concentration on neck blast incidence							
1. Co39 2. IRBL1 3. NSIC Rc216	1. 2.5 x 10^4 2. control (0)	Injection	0.5	25G x 5/8” 0.50 mm x 16 mm	Upward	Booting	Split plot with 3 replications Main plot: Variety Sub plot: Inoculum concentration
Experiment 3. Effect of variety and inoculum concentration at heading stage on neck blast incidence and incubation period							
1. Co39 2. NSIC Rc216	1. 5 x 10^4 2. 1 x 10^5 3. 2 x 10^5 4. 0 (Control)	Injection	0.5	25G x 5/8” 0.50 mm x 16 mm	Upward	Heading	Split plot with 3 replications Main plot: Variety Sub plot: Inoculum concentration
Experiment 4. Effect of inoculum concentration and syringe needle size used for injection of inoculum on neck blast incidence, lesion length, and incubation period							
Co39	1.. 1 x 10^4 2. 1.5 x 10^4 3. 2 x 10^4 4. 0 (Control)	Injection	0.5	1. 0.25 mm x 8 mm (31G, hypodermic syringe) 2. 0.50 mm x 16 mm 25G x 5/8” (disposable syringe)	Upward	Flowering	Split plot with 3 replications Main plot: Inoculum concentration Sub plot: Needle size
Experiment 5. Effect of inoculum concentration and direction of syringe during injection on neck blast incidence and incubation period							
Co39	1. 1 x 10^4 2. 2.5 x 10^4 3. 5 x 10^4 4. 1 x 10^5 5. 0 (control)	Injection	0.5	0.25 mm (31G) x 8 mm	1. Downward 2. Upward	Flowering	Split plot with 3 replications Main plot: Inoculum concentration Sub plot: Syringe direction during injection

Table 1. Summary of experimental treatments for the development of the method for neck blast inoculation.

Variety	Inoculum concentration (spore/ml)	Delivery system: Delivery method	Injection method: Vol. (ml)	Injection method: Needle size	Injection method: Direction of syringe	3. Plant growth stage	Experimental design
Experiment 6. Evaluation of the effect of inoculum concentration, growth stage, and delivery volume during injection on neck blast incidence and incubation period							
Co39	1. 2.5 x 10^4 2. 5 x 10^4 3. 0 (control)	Injection	1. 0.3 2. 0.5	0.25 mm (31G) x 8 mm	Downward	1. Booting 2. Heading	Split-split plot with 3 replications Main plot: Inoculum concentration Sub plot: Growth stage Sub-sub plot: Inoculum volume
Experiment 7. Evaluation of concentration of inoculum and heading percentage during injection on neck blast incidence and incubation period							
NSIC Rc216	1. 1 x 10^4 2. 5 x 10^4 3. 0 (control)	Injection	0.3	0.25 mm (31G) x 8 mm	Downward	1. 25% Heading 2. 50% Heading	Split plot with 3 replications Main plot: Inoculum concentration Sub plot: Heading Percentage
Experiment 8. Effect of Inoculation Delivery Methods on Neck Blast Incidence and Lesion Length							
Co39	2.5 x 10^4	1. Spraying 2. Brushing 3. Cotton without neck pricking 4. Cotton with neck pricking 5. Injection	0.3	0.25 mm (31G) x 8 mm	Downward	Flowering	RCBD with 3 replications
Experiment 9. Effect of Variety, Inoculum Concentration, and Inoculation Method on Neck Blast Incidence and Lesion Length							
1. Co39 2. NSIC Rc216	1. 0 (control) 2. 2.5 x 10^4 3. 1 x 10^5	1. Cotton with neck pricking 2. Injection	0.3	0.25 mm (31G) x 8 mm	Downward	Early Flowering	Split-split plot with 3 replications Main plot: Variety Sub plot: Inoculum concentration Sub-sub plot: Inoculation method
Experiment 10. Effect of Variety, Inoculum Concentration, and Inoculation Method on Neck Blast Incidence, Incubation Period, Lesion Length, and Relative Lesion Length							
1. Co39 2. NSIC Rc216	1. 0 (control) 2. 2.5 x 10^4 3. 1 x 10^5	1. Injection 2. Cotton without neck pricking 3. Cotton with neck pricking	0.3	0.25 mm x 8 mm (31G)	Downward	Early Flowering	Split-split plot with 3 replications Main plot: Variety Sub plot: Inoculum concentration Sub-sub plot: Inoculation method

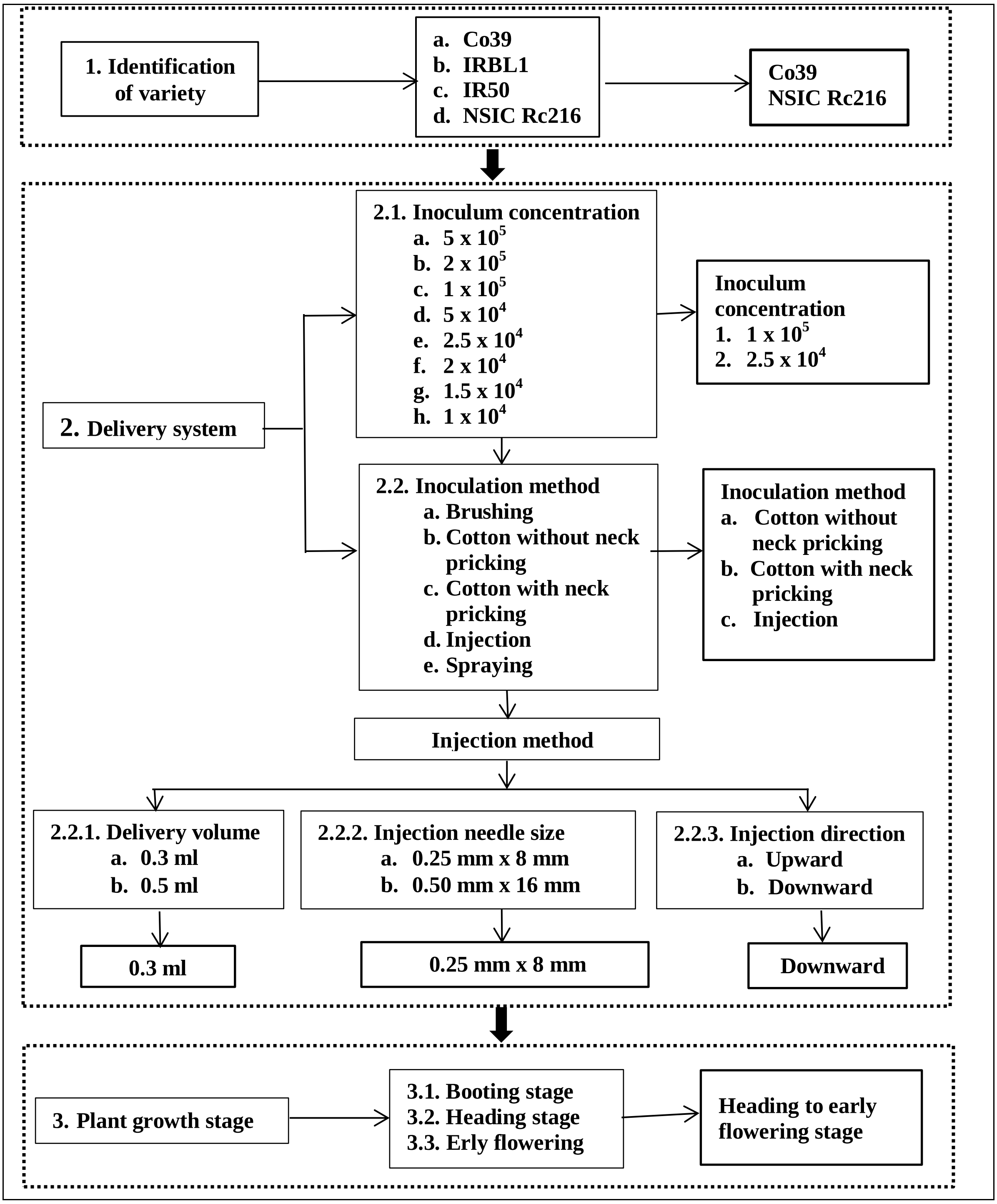

Figure 7. Flow diagram for the development of the neck blast inoculation method based on different experiments.

Results

Symptoms observed on artificially inoculated necks closely resembled those observed in naturally infected plants. Typical symptoms included reddish-brown to grayish-brown and black lesions on the neck (Figure 8A). Although less frequent, infected panicles broke at the infected neck and eventually fell to the ground (Figure 8B). Neck blast also led the formation of whiteheads, which are characterized by partially filled or unfilled spikelets (Figure 8C). Severely infected plants displayed either partially or completely rotten panicles.

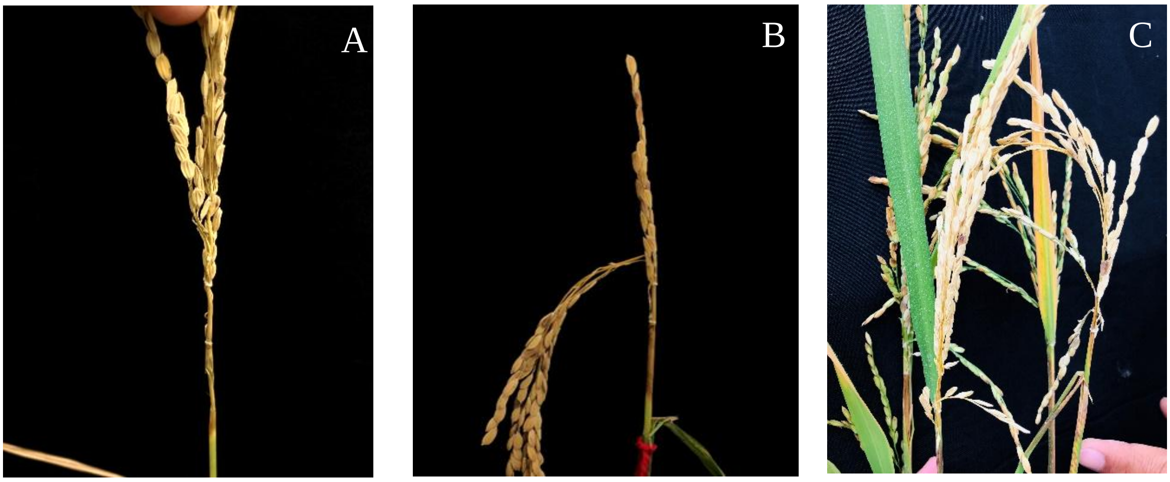

Figure 8. The neck blast symptoms on inoculated neck. A. Inoculated neck with brown to gray lesion and partially filled spikelets, B. with lesion and spikelets detached from the panicle, and (C) with whitehead (whitish filled and partially filled spikelets)

Experiment 1. Analysis of the effect of variety, inoculation method, and plant growth stage on neck blast incidence

Results of Experiment 1 showed that variety affected neck blast incidence. At 14 dai, neck blast incidence on Co39 did not significantly differ from that on IR50 but was significantly higher than on NSIC Rc216 (Table 2). The interaction between variety and injection method, as well as between variety and growth stage, did not significantly affect neck blast incidence. This indicates that the effect of variety on neck blast incidence was consistent across different injection methods and growth stages (Figure 9).

Injecting the inoculum into the neck resulted in a significantly higher neck blast incidence compared to spraying, with the incidence being four times greater than that of the spraying method. It was observed that the effect of inoculation method varied according to the growth stage. The difference in neck blast incidence between inoculation methods was more pronounced at the heading stage than at the late booting stage. During the heading stage, the incidence of neck blast was about 30 times higher with the injection method compared to the spraying method. In contrast, at the late booting stage, the incidence using this method was 20 times higher.

Table 2. Effect of variety, inoculation method, and growth stage on neck blast incidence at 14 days after inoculation in Experiment 1.

Factor and treatment*	Neck blast incidence (%)
Variety	
IR50	25.83 ab
Co39	35.00 a
NSIC RC 216	20.83 b
Inoculation method	
Spraying	1.11 b
Injection	53.33 a
Growth stage	
Late booting	10.00 b
Heading	44.44 a

*Within a factor, treatment means with the same letter are not significantly different using Fischer's Least Significant Difference (LSD) test.

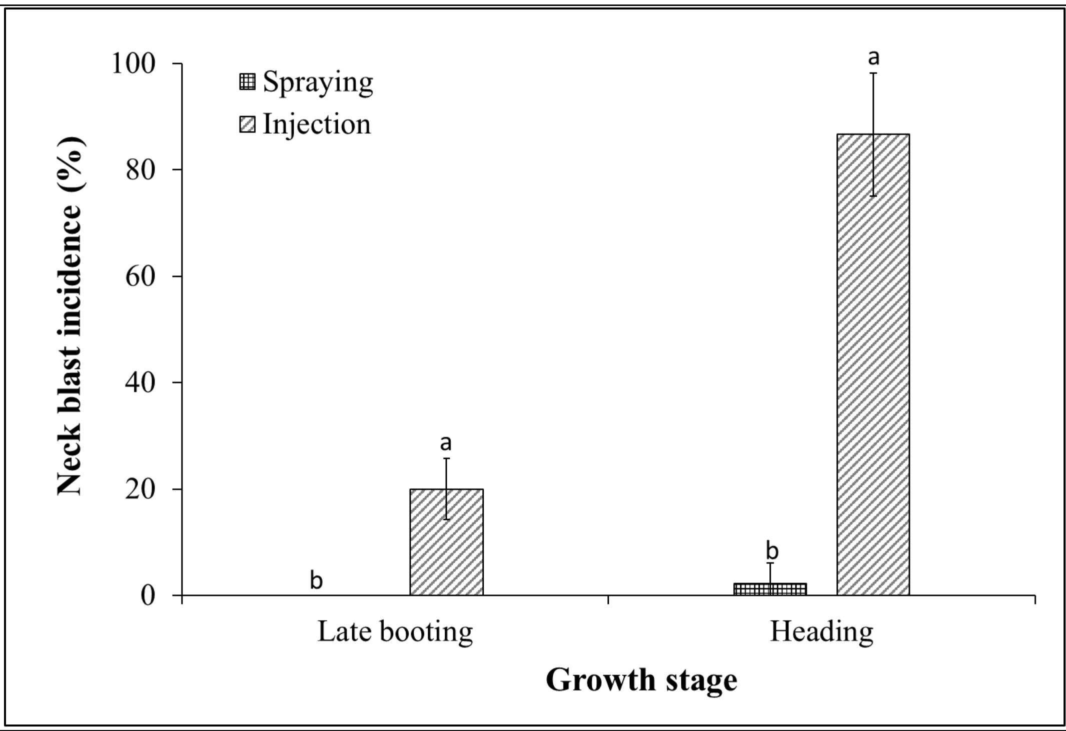

Figure 9. Effect of inoculation method and growth stage during inoculation on neck blast incidence, Experiment 1. In a growth stage, means followed by the same letter are not significantly different at p<0,05 using LSD test.

Experiment 2. Analysis of the effect of variety and inoculum concentration on neck blast incidence.

Neck blast incidence on the tested varieties did not show significant differences (Table 3,) even though previous studies indicated that these varieties varied in their susceptibility to the disease. However, injecting the neck with a concentration of 2.5 x 10^4 spores/ml resulted in a higher neck blast incidence compared to the control.

Table 3. Effect of variety and inoculum concentration on neck blast incidence, Experiment 2.

Factor and treatment*	Neck blast incidence (%)	
Variety		
Co39	48.33	a
NSIC Rc216	45.00	a
IRBL1	43.33	a
Inoculum concentration		
Control (0)	0.00	b
2.5 x 10^4 spores per ml	91.11	a

*Within a factor, treatment means with the same letter are not significantly different using LSD test.

Experiment 3. Analysis of the effect of variety, and inoculum concentration at heading stage on neck blast incidence and incubation period

In Experiment 3, neither the variety (Co39 and NSIC Rc216) nor its interaction with inoculum concentration significantly affected the incidence of neck blast (Table 4). However, plants injected with 2.5 x 10^5 spores/ml showed a significantly higher neck blast incidence compared to those injected with concentrations of 5 x 10^4, and 1 x 10^5 spores/ml. Neck blast was not observed in necks injected with sterile water (Table 4). In addition, the incubation period for NSIC Rc216 was significantly longer than that for Co39 (Table 4). The concentration of the spore suspension also significantly influenced the incubation period. In decreasing order, the incubation periods at different spore concentrations were 5 x 10^4, 1 x 10^5, 2 x 10^5, and the control.

Table 4. Effect of variety and inoculum concentration on neck blast incidence and incubation period at 14 days after inoculation, Experiment 3.

Factor and treatment*	Neck blast incidence (%)		Incubation period (day)	
Variety				
Co39	70.00	a	4.10	b
NSIC Rc216	63.33	a	4.33	a
Inoculum concentration (spore/ml)				
0	0.00	c	0.00	d
5×10^4	80.00	b	6.67	a
1×10^5	86.67	b	5.60	b
2×10^5	100.00	a	4.60	c

*Within a factor, treatment means with the same letter are not significantly different using LSD test.

Experiment 4. Analysis of the effect of inoculum concentration and syringe needle size used for injection of inoculum on neck blast incidence, lesion length, and Incubation period

In Experiment 4, inoculum concentration and size of syringe needle did not significantly affect neck blast incidence, lesion length, and incubation period (Table 5).

Table 5. Effect of inoculum concentration and syringe needle size on neck blast incidence, lesion length, and incubation period at 28 days after inoculation, Experiment 4.

Factor and treatment*	Neck blast incidence (%)	Lesion length (cm)	Incubation period (day)
Inoculum concentration			
1.0×10^4	86.94 a	6.34 a	8.40 a
1.5×10^4	100.00 a	6.38 a	8.12 a
2.0×10^4	100.00 a	7.42 a	8.22 a
Needle size			
0.25 mm x 8 mm	93.52 a	7.02 a	8.16 a
0.50 mm x 16 mm	97.78 a	6.41 a	8.34 a

* In a factor, treatment means with the same letter are not significantly different at $p < 0.05$ using LSD test.

Experiment 5. Effect of inoculum concentration and direction of syringe during injection on neck blast incidence and incubation period

The results of Experiment 5 showed that the concentration of spore suspension ranging from 2.5×10^4, 5×10^4, and 1×10^5 spores/ml significantly affected neck blast incidence and incubation period (Table 6). Neck blast incidence increased significantly with higher concentrations of spore suspension. However, the incubation period showed no significant difference between 1 x 10^4 and 2.5 x 10^4 spores/ml, and it decreased as the concentration of the spore suspension increased. The direction of the syringe during inoculation did not affect neck blast incidence. Incubation period was significantly longer when the syringe was positioned upward at 15° than when positioned downward at the same angle (Table 6).

Table 6. Effect of the amount of inoculum and direction of syringe during inoculation on neck blast incidence and incubation period, Experiment 5.

Factor and treatment*	Neck blast incidence (%)		Incubation period (day)	
Inoculum concentration (spore/ml)				
1 x 10^4	60.00	d	7.67	a
2.5 x 10^4	81.67	c	7.00	ab
5 x 10^4	88.33	b	6.17	b
1 x 10^5	100.00	a	5.00	c
Control	0.00	e	0.00	d
Syringe direction during injection				
Upward	62.00	a	4.67	b
Downward	70.00	a	5.67	a

*In a factor, treatment means with the same letter are not significantly different at $p \leq 0.05$ using LSD test.

Experiment 6. Effect of inoculum concentration, growth stage, and delivery volume during injection on neck blast incidence and incubation period

The results of Experiment 6 indicate that, at 21 dai, both the concentration of inoculum and growth stage significantly affected neck blast incidence (Table 7), The interaction between inoculum concentration and growth stage during inoculation also had a significant effect on neck blast incidence, indicating that the effect of spore concentration significantly varied depending on the growth stage. At booting stage, injecting the necks of plants with 5 x 10^4 spores/ml resulted in a higher incidence of neck blast compared to 2.5 x 10^4 spores/ ml (Figure 10). In contrast, there was no significant difference in neck blast incidence between the two concentrations of spore suspension at heading stage.

The incubation period did not show a significant difference between inoculations using 2.5 x 10^4 spores/ml and 5 x 10^4 spores/ml. However, it was significantly longer when inoculation was done at heading stage compared to booting stage (Table 7). The volume of inoculum had no effect on neck blast incidence and incubation period.

Table 7. The effect of the amount of inoculum, growth stage, and delivery volume on neck blast incidence and incubation period at 21 days after inoculation in Experiment 6.

Factor and treatment*	Neck blast incidence (%)	Incubation period (days)
Inoculum concentration, spore per ml		
2.5×10^4	53.33 b	9.25 a
5×10^4	63.33 a	7.58 a
Control	0.00 c	0.00 b
Growth stage		
Booting	24.44 b	6.94 b
Heading	53.33 a	4.28 a
Delivery volume, ml		
0.3	37.78 a	5.83 a
0.5	40.00 a	5.39 a

*In a factor, treatment means with the same letter are not significantly different at $p \leq 0.05$ using LSD test.

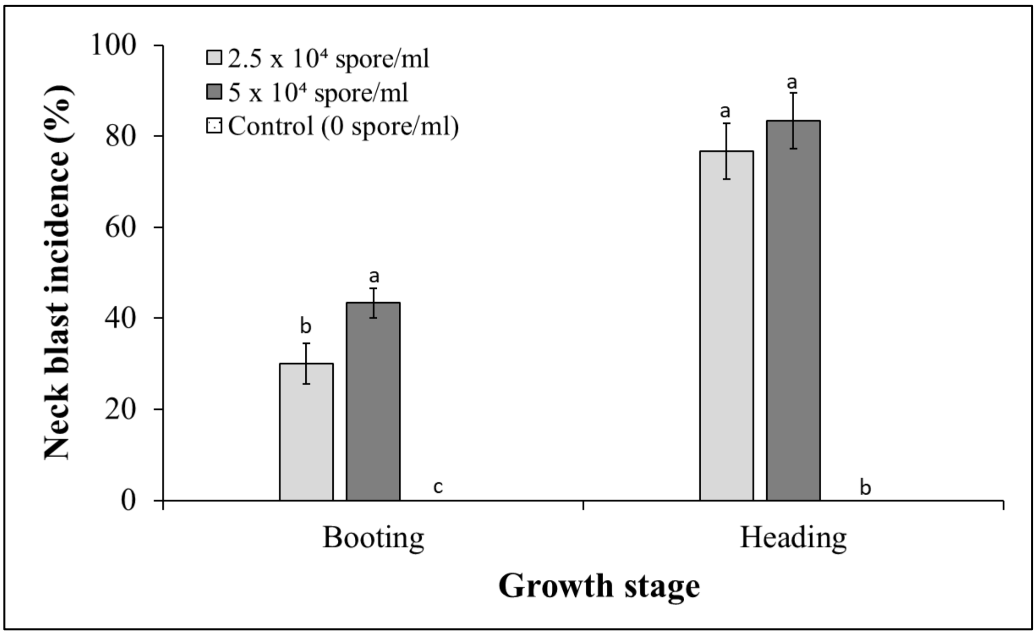

Figure 10. Effect of inoculum concentration and growth stage on the neck blast incidence inoculation, Experiment 6. In a growth stage, means followed by the same letter are not significantly different at *p*<0,05 using LSD test.

Experiment 7. Evaluation of concentration of inoculum and heading percentage during injection on neck blast incidence and incubation period

In Experiment 7, the effects of the spore suspension concentration and heading percentage (proportion of tillers in a pot that have reached the heading stage) on neck blast incidence and incubation period were analyzed. The concentration of spore suspension significantly affected neck blast incidence with the concentration of 5 x 10^4 spores/ml having higher neck blast incidence compared to 1 x 10^4 spores/ml (Table 8). However, no significant difference in neck blast incidence was observed between the 25% and 50% heading percentages.

Incubation period was shorter with the application of spore concentration of 5 x 10^4 spores/ml compared to 1 x 10^4 spores/ml, and when spore suspension was applied at 50% heading compared to 25% heading. (Table 8). The interaction between inoculum concentration and heading percentage significantly affected the incubation period (Figure 11). However, the effect of heading percentage on the incubation period was consistent in inoculated plants, showing a shorter period as the inoculum concentration increased. In contrast, there was no difference observed in the control group, where the plants were injected with sterile water.

Table 8. The effect of the concentration of inoculum and growth stage on neck blast incidence and incubation period at 21 days after inoculation in Experiment 7.

Factor and treatment*	Neck blast incidence (%)	Incubation period (days)
Inoculum concentration		
1 x 10^4	55.55 b	11.33 a
5 x 10^4	83.33 a	8.17 b
Control (0)	0.00 c	0.00 c
Heading percentage		
25% heading	40.74 a	7.78 a
50% heading	51.85 a	5.22 b

*In a factor, treatment means with the same letter are not significantly different at $p \leq 0.05$ using LSD test.

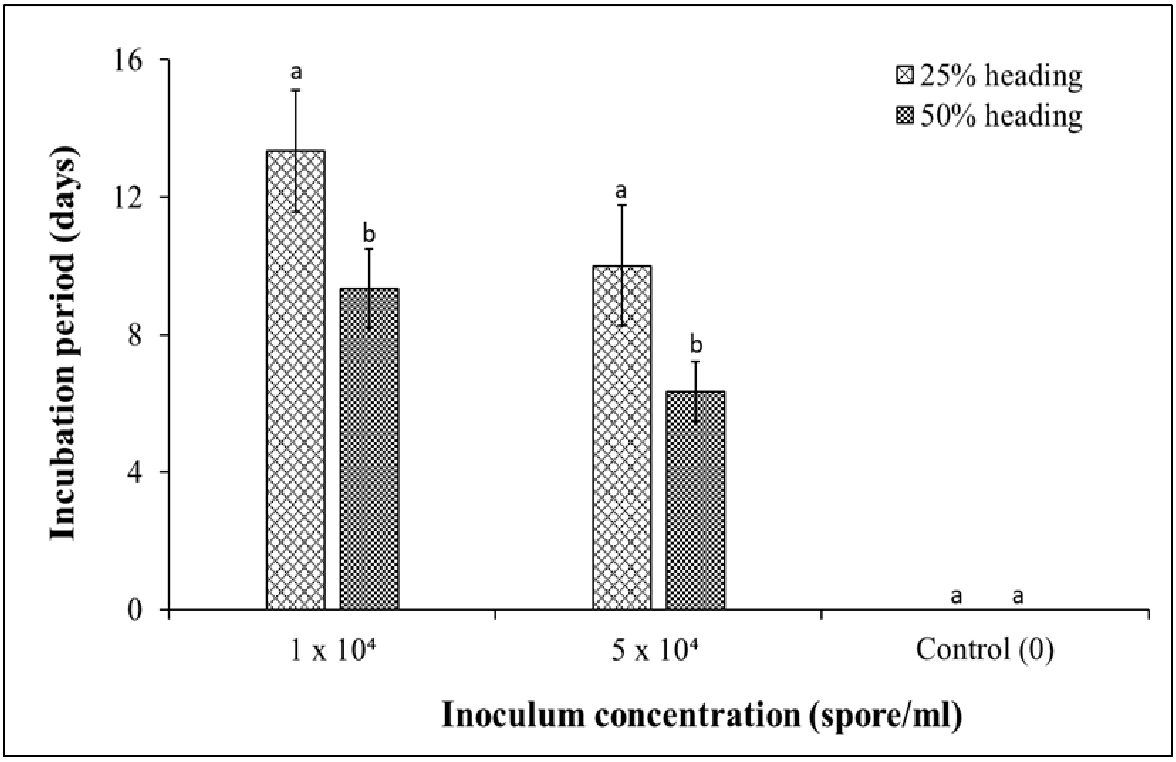

Figure 11. Effect of inoculum concentration and heading percentage on incubation period, Experiment 7. In a growth stage, means followed by the same letter are not significantly different at $p<0{,}05$ using LSD test.

Experiment 8. Effect of inoculation delivery methods on neck blast incidence and lesion length

The different inoculated methods tested in Experiment 8 were injection of spore suspension, spraying of spore suspension, brushing the neck with spore suspension, wrapping the neck with cotton soaked in spore suspension, wrapping the pricked neck with cotton soaked in spore suspension, and injection of sterilized water.

The inoculation method significantly affected neck blast incidence at 10, 18, and 24 dai (Figure 12). The highest incidence of neck blast occurred with the injection of spore suspension into the neck, followed by wrapping the pricked neck with cotton soaked in spore suspension (Figure 13). The incidence of neck blast using the other inoculation methods was lower and did not significantly differ from each other. Neck blast incidence on injected plants was 86.67% from 10 to 24 dai, while the incidence on plants with pricked necks wrapped in cotton soaked in spore suspension was recorded at 27.41% at 10 dai and 37.38% at both 18 and 24 dai. In contrast, neck blast was not observed on necks that were sprayed with spore suspension or injected with sterile water.

The injection method consistently produced significantly longer lesion length than the other methods at 10, 18, and 24 dai (Figure 14). Lesion length increased from 10 to 24 dai on necks injected with spore suspension. Although lesion length also increased in pricked necks wrapped with cotton soaked in spore suspension, the length was shorter and the rate of increase was slower compared to the injection method.

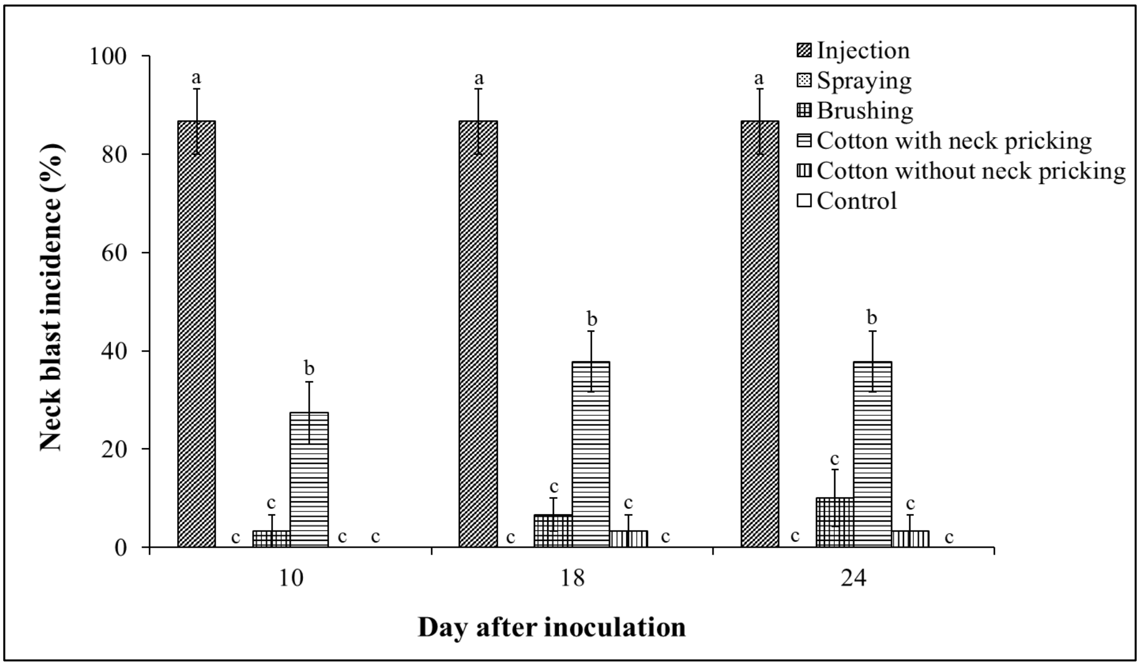

Figure 12. Effect of inoculation method on neck blast incidence at 10, 18, and 24 days after inoculation, Experiment 8. In a day after inoculation, bars with the same letter are not significantly different at *p*<0,05 using LSD test.

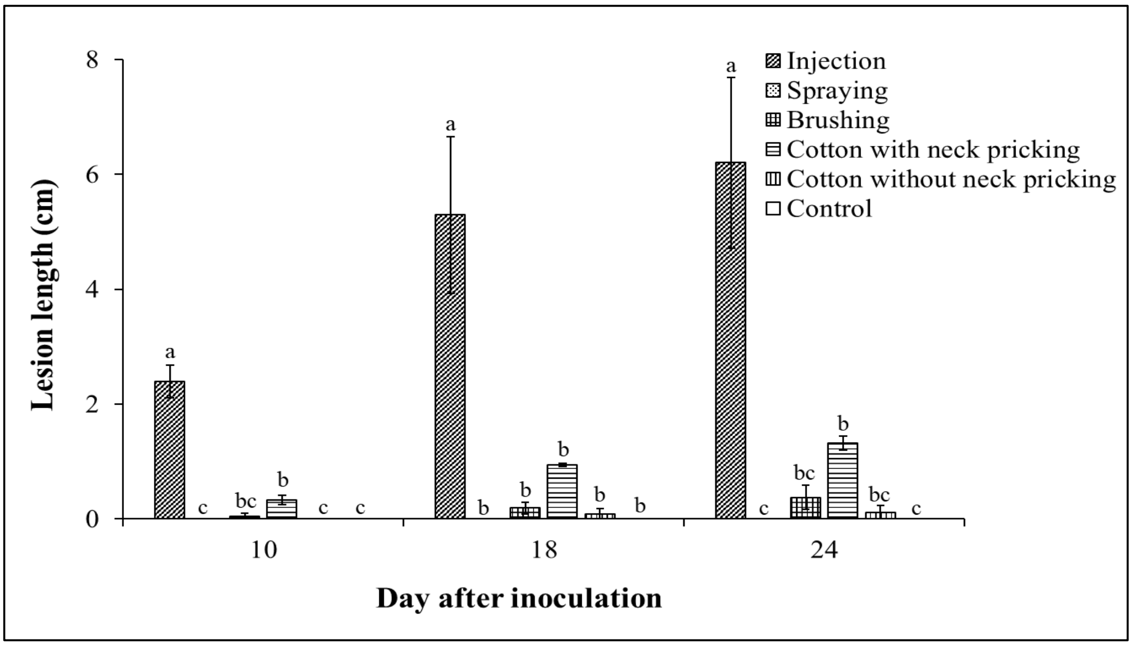

Figure 13. Effect of inoculation method on lesion length at 10, 18, and 24 days after inoculation, Experiment 8. In a day after inoculation, bars with the same letter are not significantly different at p<0,05 using LSD test.

Experiment 9. Effect of variety, inoculum concentration, and inoculation method on neck blast incidence and lesion length

The effect of varieties (Co39 and NSIC Rc216), inoculation methods (injection and wrapping pricked neck with cotton soaked in spore suspension) and concentrations (2.5 x 10^4 and 1 x 10^5 spores/ ml) that were found to have the highest efficacy in previous experiments were analyzed in Experiment 9. As observed in Experiment 8, neck blast incidence was significantly higher when the spore suspension was injected into the neck than when cotton is mixed with the suspension and wrapped around the pricked neck (Appendix 9a, Table 10). However, the effect of inoculation method varied according to the inoculum concentration and vice versa. As shown in Figure 14, neck blast incidence did not differ in injected plants. However, when the cotton with neck pricking method was used, the inoculum concentration of 1 x 10^5 spores/ml resulted in higher neck blast incidence (74.74 %,) than 2.5 x 10^4 spores per ml (53.33 %).

Inoculation method, inoculum concentration and their interaction also affected lesion length (Table 9). Overall, the injection method resulted in longer lesion length compared to the cotton with neck pricking method. However, as shown in Figure 15, spore concentration did not affect the lesion length on injected plants, but when the cotton with neck pricking method was used, lesion length was longer at inoculum concentration of 1 x10^5 spores/ml than at 2.5 x 10^4 spores/ml. Variety did not affect neck blast incidence and lesion length.

Table 9. The effect of variety, inoculum concentration, and injection method on neck blast incidence and incubation period, Experiment 9.

Factor and treatment*	Neck blast incidence (%)		Lesion length (cm)	
Variety				
Co39	42.69	a	5.58	a
NSIC Rc216	37.78	a	5.62	a
Inoculum concentration (spore/ml)				
0	0.00	c	0.00	b
2.5 x 10^4	50.83	b	8.32	a
1 x 10^5	69.87	a	8.48	a
Inoculation method				
Injection	63.89	a	10.84	a
Cotton with neck pricking	16.58	b	0.35	b

*In a factor, treatment means with the same letter are not significantly different at $p \leq 0.05$ using LSD test.

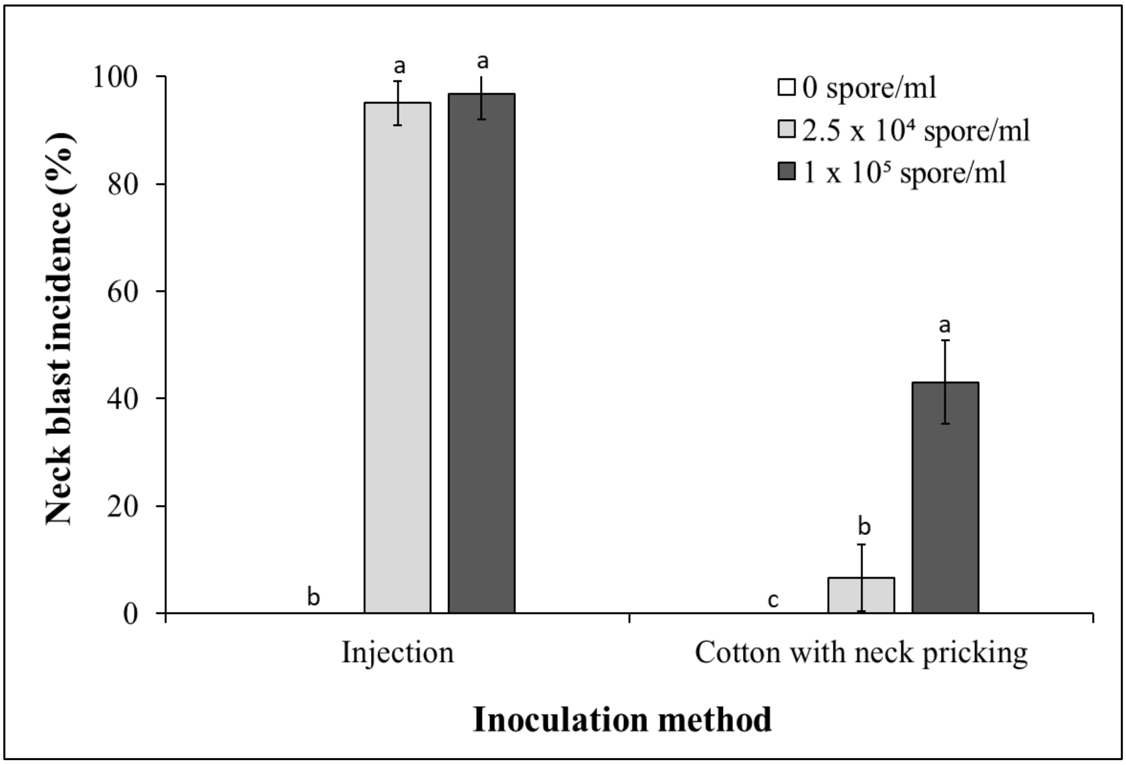

Figure 14. Effect of inoculum concentration and inoculation method on the neck blast incidence, Experiment 9. In an inoculation method, bars with the same letter are not significantly different at *p*<0,05 using LSD test.

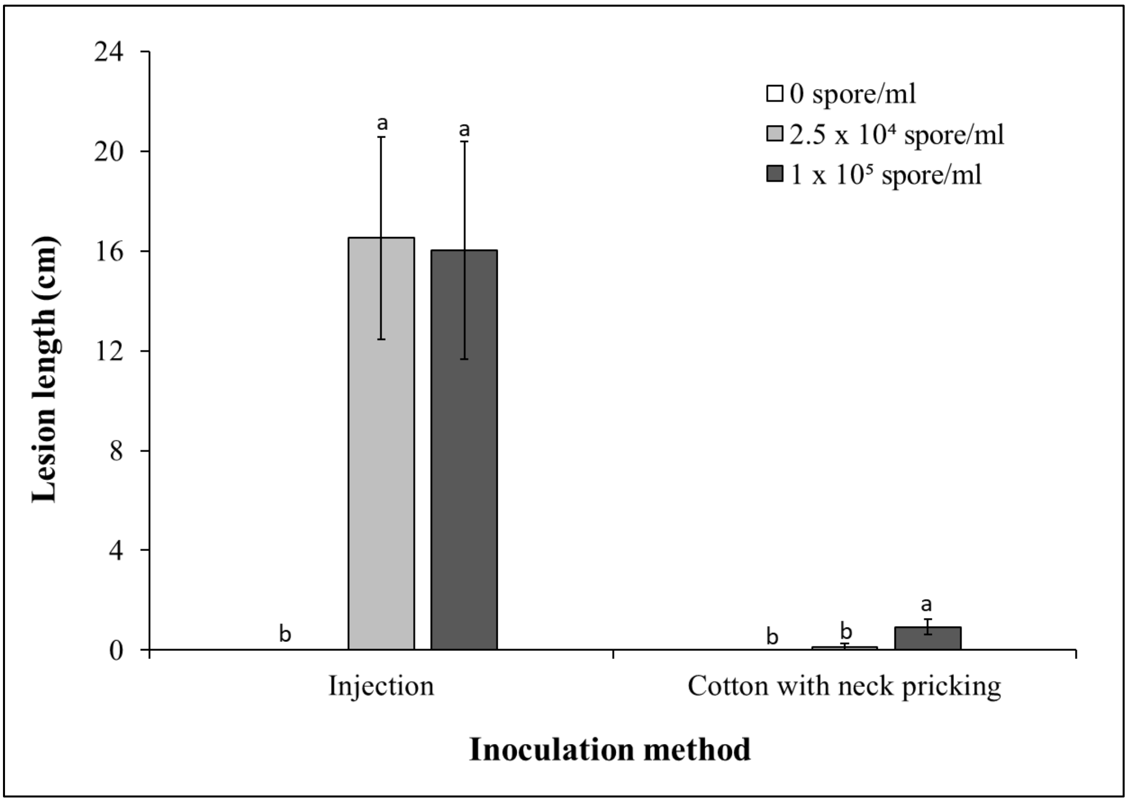

Figure 15. Effect of inoculum concentration and inoculation method on lesion length, Experiment 9. In an inoculation method, bars with the same letter are not significantly different at *p*<0,05 using LSD test.

Experiment 10. Effect of variety, inoculum concentration, and inoculation method on neck blast incidence, incubation period, lesion length, and relative lesion length

Experiment 10 was conducted to confirm the results obtained in Experiments 8 and 9 to optimize the components for inoculating *M. oryzae* into the neck. The effects of variety (Co39 and NSIC Rc216), inoculation method (injection and cotton with and without neck pricking) and inoculum concentration (2.5 x 10^4 and 1 x 10^5 spores/ml) on four disease variables (neck blast incidence, incubation period, lesion length and relative lesion length) were analyzed in this experiment.

As observed in Experiments 8 and 9, injection method, inoculum concentration and their interaction affected neck blast incidence (Table 10, Figure 16). In decreasing order, the incidence of neck blast associated with different inoculation methods was highest for the injection method, followed by the cotton with pricking method, and lowest for the cotton without pricking method (Table 10). Figure 16 shows that the concentration of 1 x 10^5 spores/ml resulted in significantly higher neck blast incidence than 2.5 x 10^4 spores per ml in all inoculated plants. However, the neck blast incidence did not differ between 2.5 x 10^4 spores per ml and the control with the use of cotton without neck pricking method.

Injection method, inoculum concentration and their interaction also affected incubation period (Table 10). Regardless of whether the neck was pricked or not, the incubation period was longer with the cotton method across all varieties and inoculum concentrations compared to the injection method. The concentration of the inoculum did not significantly affect the incubation period when using the injection method or the cotton without neck pricking method. However, a spore concentration of 2.5 x 10^4 spores per ml led to a longer incubation period compared to the concentration of 1 x 10^5 spores per ml when the cotton without neck pricking method was used (Figure 17).

Variety affected lesion length and relative lesion length, but the effect varied according to the inoculation method. When the injection method was used, lesion length and relative lesion length were significantly longer on Co39 than NSIC Rc216 (Figures 18 and 19, respectively). However, variety had no effect on both disease variables when cotton was used regardless of whether the neck was pricked or not. The effect of inoculum concentration on lesion length and relative lesion length varied according to the inoculation method. The injection method produced greater lesion length and relative lesion length compared to the cotton-based methods (Table 10). Additionally, these measurements were consistently higher at a concentration of 1 x 10^5 spores per ml than at 2.5 x 10^4 spores per ml across all inoculation methods (Figures 20 and 21).

The results of all the experiments showed that the most effective method to inoculate the neck with *M. oryzae* involves injection of spore suspension into the neck of Co39 variety at heading stage. Although heading stage produced high neck blast incidence, inoculation at flowering stage is also recommended because more panicles have existed at this stage and thus NSIC Rc216 can be used as an alternative to Co39 because its performance in some experiments matches that of Co39, and its morphology and growth duration is similar to the varieties currently cultivated by farmers. During injection, a hypodermic needle sized 0.25 mm x 8 mm should be used and its position should be at a downward angle (15 to 20°). The concentration of the spore suspension during injection should be 1 x 10^5 spores/ml, with a volume of 0.3 ml.

Table 10. The effect of variety, inoculum concentration, and inoculation method on neck blast incidence, incubation period, lesion length, and relative lesion length, Experiment 10.

Factor and Treatment*	Neck blast incidence (%)		Incubation period (day)		Lesion length (cm)		Relative lesion length (%)	
Variety								
Co39	29.26	a	6.98	a	4.89	a	9.36	a
NSIC Rc216	27.78	a	7.94	a	3.74	a	5.52	a
Inoculum concentration (spore/ml)								
0	0.00	c	0.00	c	0.00	c	0.00	c
2.5 x 10^4	32.22	b	11.95	a	5.12	b	8.85	b
1 x 10^5	53.33	a	10.43	b	7.84	a	13.46	a
Inoculation method								
Injection	59.44	a	4.92	b	9.57	a	16.64	a
Cotton without neck pricking	8.33	c	9.06	a	1.49	b	2.45	b
Cotton with neck pricking	17.78	b	8.41	a	1.89	b	3.22	b

*In a factor, treatment means with the same letter are not significantly different at $p < 0.05$ using LSD test.

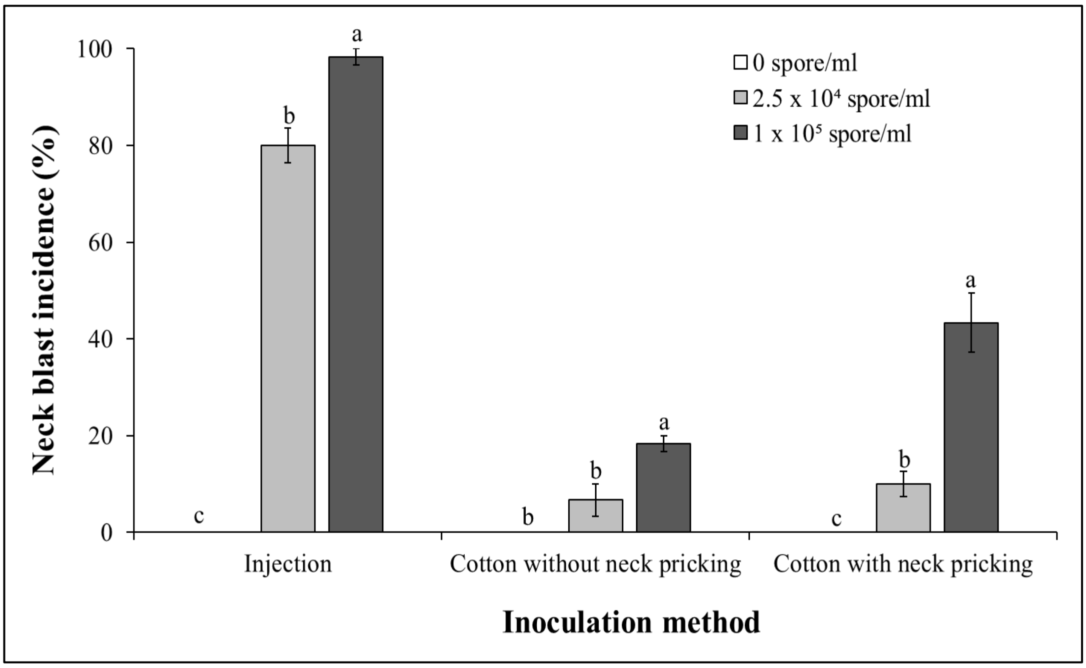

Figure 16. Effect of inoculum concentration and inoculation method on neck blast incidence, Experiment 10. In an inoculation method, bars with the same letter are not significantly different at *p*<0,05 using LSD test.

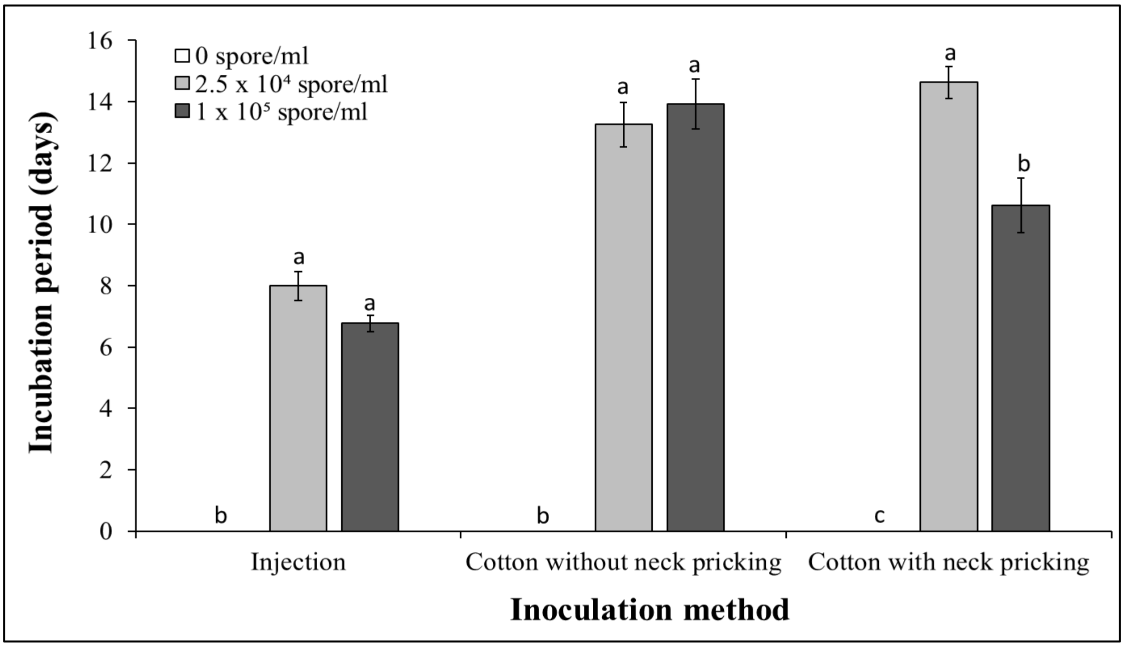

Figure 17. Effect of inoculum concentration and inoculation method on incubation period, Experiment 10. In an inoculation method, bars with the same letter are not significantly different at *p*<0,05 using LSD test.

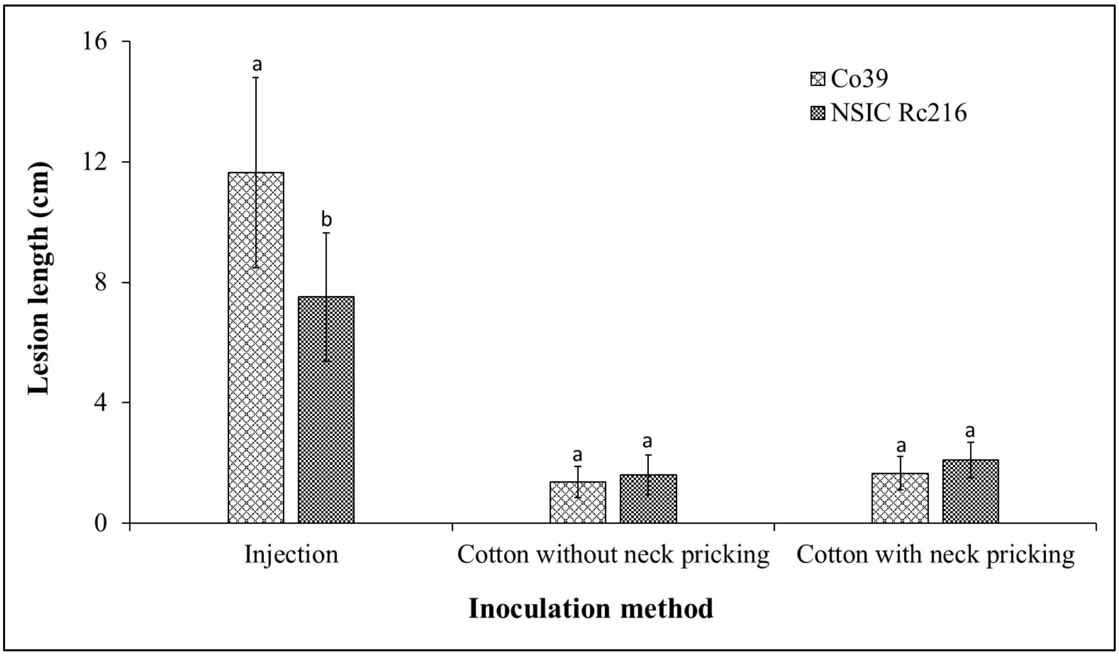

Figure 18. Effect of variety and inoculation method on lesion length, Experiment 10. In an inoculation method, bars with the same letter are not significantly different at *p*<0,05 using LSD test.

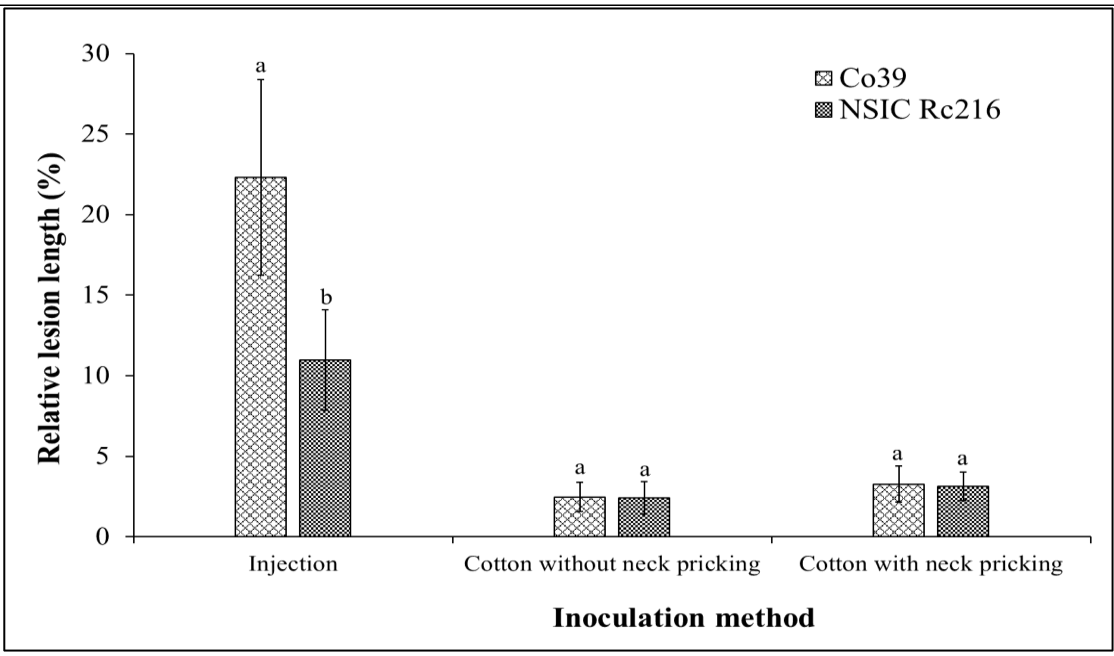

Figure 19. Effect of variety and inoculation method on relative lesion length, Experiment 10. In an inoculation method, bars with the same letter are not significantly different at *p*<0,05 using LSD test.

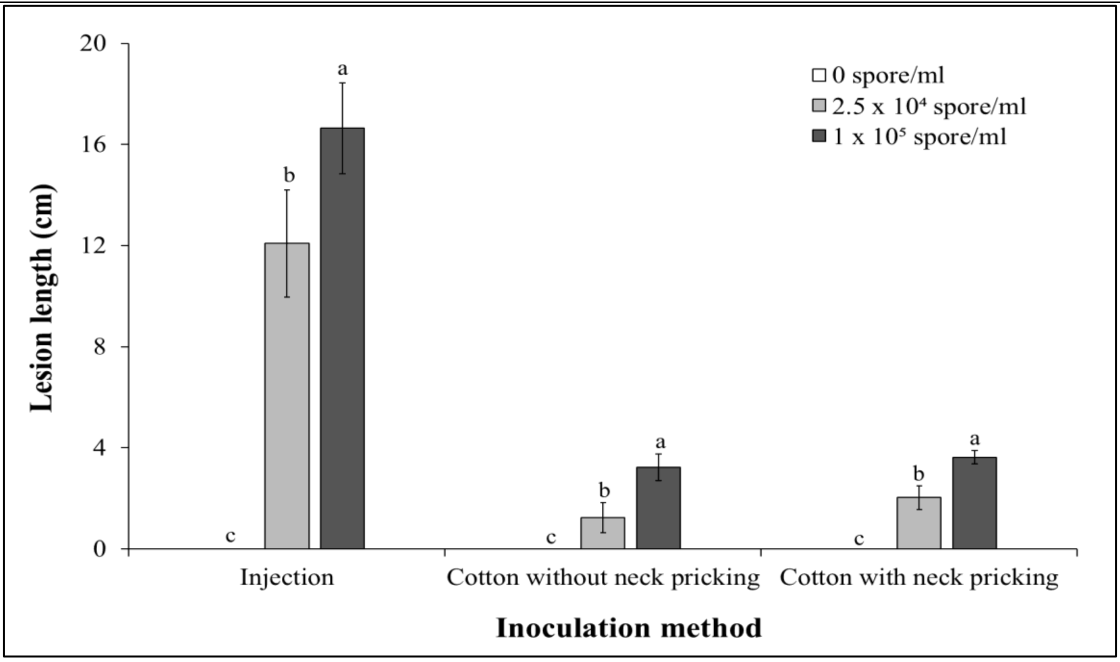

Figure 20. Effect of the inoculum concentration and inoculation method on lesion length, Experiment 10. In an inoculation method, bars with the same letter are not significantly different at p<0,05 using LSD test.

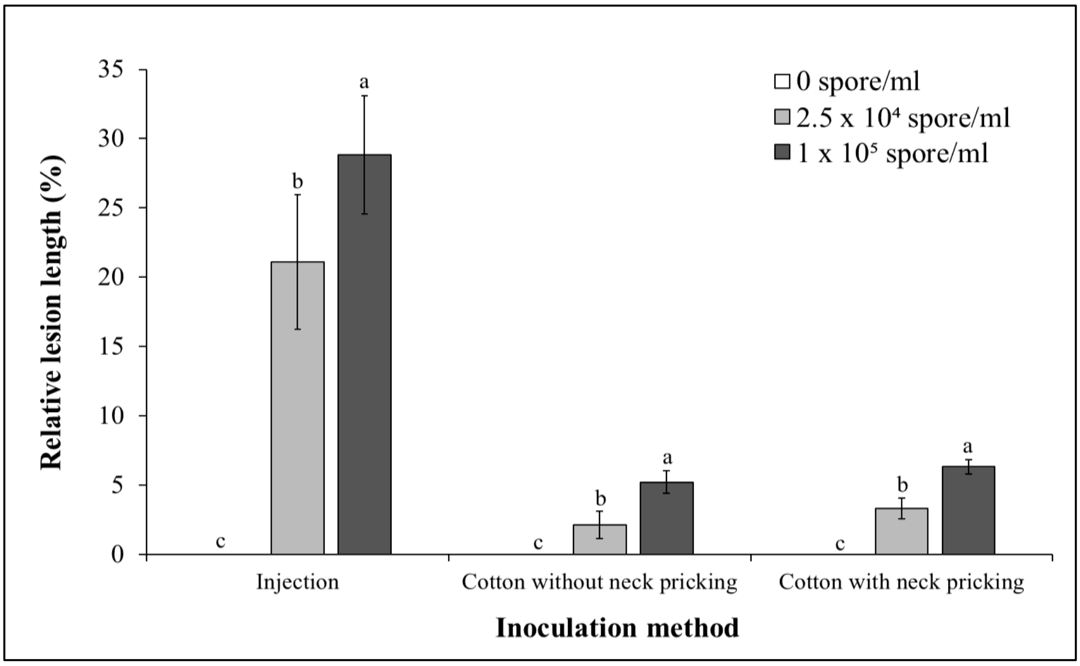

Figure 21. Effect of the inoculum concentration and inoculation method on relative lesion length, Experiment 10. In an inoculation method, bars with the same letter are not significantly different at p<0,05 using LSD test.

Discussion

The inoculation methods tested in this study were selected based on their reported effectiveness and reliability in producing neck blast and other plant diseases under controlled conditions. The method of spraying the spore suspension of the blast pathogen to the target plant tissues is widely used due to its convenience, reproducibility (Chen et al., 1995), ability to mimic natural infection processes and the actual route of pathogen infection (Imathiu et al., 2014), and suitability for large-scale tests. Spraying is less labor-intensive compared to point inoculation methods tested in this study, specifically the injection, cotton, and brushing methods. Spraying of rice leaves is a common practice for producing leaf blast (Chen et al., 1985). The injection method allows for direct delivery of the inoculum to the neck, minimizing wastage. Moreover, plant injury caused by this method is known to facilitate pathogen establishment (Clements et al., 2003). Using cotton dipped in spore suspension allows for efficient inoculum application, as the suspension adheres to the neck, minimizing wastage compared to spraying and brushing, where some inoculum may be lost due to drift or runoff. It also maintains moisture of inoculated plant tissues (Grausgruber et al., 1995). The non-invasive techniques of using cotton without pricking the neck, along with spraying and brushing, were tested as they reduce the risk of physically damaging plant tissues during application and are relatively easy to use.

The needle size for the injection method was tested in this study because previous studies indicated that choosing the right needle size is crucial for balancing effective delivery of inoculum and minimizing damage to plant tissues. Small needle size may restrict the flow of the suspension, which may lead to clogging if the spore suspension is not well-filtered and have mycelial fragments of *M. oryzae*. Smaller needle size allows more precise injections and minimizes damage, particularly when used in small or delicate tissues such as the neck. In contrast, larger needle.

The series of experiments conducted at IRRI led to the identification of the components necessary for an inoculation method to introduce *M. oryzae* into the neck. Among the varieties tested, Co39 and NSIC Rc216 were the most suitable because of their high susceptibility to neck blast. However, although Co39 is widely used as susceptible control in studies involving blast (Jiang et al., 2019; Greenwood and Glaus, 2022) and had higher neck blast incidence than NSIC Rc216 in some experiments, its growth duration is shorter than rice varieties that are typically grown by farmers. In contrast, NSIC Rc216 has a growth duration and morphology that are more representative of the currently popular inbred varieties and could therefore be considered as the host for artificial inoculation of the pathogen for epidemiological studies.

Results from Experiments 1, 8, 9, and 10 consistently demonstrated that the injection method is most effective for artificially inoculating *M. oryzae* into the neck, supporting the results of other studies (Bhaskar et al, 2017). This method produced the highest incidence of neck blast, the shortest incubation period, and both greater lesion length and relative lesion length compared to the other tested methods. The high efficacy of injection method followed by cotton soaked in spore suspension with pricking of the neck compared to the other methods indicates the need to mechanically injure the neck to successfully inoculate *M. oryzae*. Pricking the neck facilitates the entry of pathogens by bypassing the natural barriers in neck tissues which serve as physical defenses against pathogens and help to slow down the infection process. Koga (1994) observed that the surface of neck tissues is covered with a thin layer of cuticle with thick-walled epidermal cells. The tissues are reinforced by sclerenchyma which consists of 3 to 5 cell layers and the hard parenchyma tissue, making the neck rigid. Injection may have also allowed *M. oryzae* to invade tissues before the defense responses are fully activated. The method may have also bypassed the surface-localized defense mechanisms initiated by a rice plant when infected by *M. oryzae* to strengthen its overall immune response (He et al., 1999; Yin et al., 2021). Another advantage of the injection method over spraying and brushing the spore suspension on the neck is that it minimized the loss of the inoculum caused by runoff, evaporation, or high temperature and, therefore, reduced the contamination of other plant organs, surrounding plants or the inoculation chamber with the pathogen. It also ensured the application of the same amount of inoculum, reducing variability in infection rates across treated plants and efficient use of inoculum because the spore suspension is delivered directly to the neck. However, injection method may bypass natural infection processes, potentially leading to results that differ from field conditions. It requires careful handling of the plant and considerable skill of the individual using the method to ensure that same inoculum volume is applied (Grausgruber et al., 1995). Moreover, the method is laborious, just like most point inoculation methods (Imathiu et al., 2014) and will be difficult to implement in large-scale experiments.

The appropriate needle size for the injection method should be considered. The needle size for the injection method should be considered. Although the needle sizes tested in this study (0.25 mm x 8 mm and 0.50 mm x 16 mm) had the same efficacy, smaller needle size is preferred because it allows higher precision in the delivery of the pathogen, minimizes inoculum spillage and reduces the risk of damaging the plant. Although direction of the syringe and volume of the inoculum during injection did not affect inoculation efficacy, the downward position and use of 0.3 ml of inoculum are preferred because these options minimize spillage, wastage of the inoculum and contamination of other plant organs compared to the upward position and use of higher volume. Further studies may consider closing the injury created by injection by applying a cream that does not harm both the plant and pathogen to help prevent the injected spore suspension from leaking out. Asiedu et al. (2024) used Vaseline cream on maize stalks injected with different *Fusarium* species. A spore suspension concentration of 1×10^5 spores/ml is recommended for artificial inoculation. Experiments 9 and 10 demonstrated that this concentration led to significantly higher neck blast incidence, as well as longer lesion length and relative lesion length, compared to a concentration of 2.5×10^4 spores/ml.

The injection method should take into account the growth stage of experimental plants. The incidence of neck blast was higher when inoculation was done at heading (when the panicle starts to emerge from the leaf sheath) than at booting stage because it mimics the natural infection process. Research conducted by Guo et al. (2024) indicated that the infection of panicles by the blast pathogen begins at the onset of heading and continues for up to 15 days thereafter. Sun et al. (1992) observed that neck blast infection was higher when the neck was inoculated at three days after heading compared to ten days after heading. Inoculation at heading favors pathogen infection and establishment because the neck is exposed and the tissues are still soft. At booting, panicles are still wrapped by leaf sheaths (Bandong and Litsinger, 2007) which may prevent the pathogen from infecting the panicles and the neck has not yet developed. The low efficacy at booting stage also suggests that successful inoculation requires direct inoculation on the neck. Figure 9 summarizes the recommended method for inoculating *M. oryzae* to the neck based on the results of ten experiments.

Future Steps

Additional experiments will be conducted in 2025 to improve the inoculation method developed in this study. The effect of growth stage on the efficacy of the injection method should be evaluated to optimize the timing of its application. The growth stages to be considered are heading, flowering and milk because infection at these stages affect neck blast incidence under natural conditions. Moreover, it was observed that compared to the heading stage, more necks could be inoculated and sampled at flowering and milk stages because panicles of healthy plants have fully exserted at these stages. Future research should also consider testing the inoculation method in field conditions and across different cropping seasons using varieties with different levels of resistance to ensure that the method reflects disease epidemiology under field conditions. The method was developed under semi-controlled conditions, where the factors influencing disease epidemiology may not accurately reflect those in actual field environments. Testing the method under field conditions allows for improvements and will ensure that it produces reliable and reproducible results.

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