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Development of inoculation method to evaluate response of cashew against shoot dieback (*Diaporthe* spp.) and gummosis disease (*Botryosphaeria* spp.)

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Cashew is an important high valued drought-tolerant plantation crop mainly cultivated in coastal states of India. However, its productivity is severely constrained by destructive fungal diseases, notably shoot dieback caused by *Diaporthe* spp. and gummosis associated with *Botryosphaeria* spp. The present study was undertaken to develop and standardize efficient inoculation methods for inducing shoot dieback and gummosis in cashew, facilitating accurate assessment of disease development and host–pathogen interactions. Wound inoculation method showed highest Area under lesion progress curve (AULPC) value in both the isolates of *Diaporthe* spp. and *Botryosphaeria* spp. as compared to stem injection method and toothpick method of inoculation. JJJDi-1 isolate of *Diaporthe* spp. produced 11.60 cm long lesion in wound inoculation method at 21DAI followed by KKPDi-1 (7.73cm), DJJDi-1 (7.37cm) and BMJDi-1 (6.88cm). Higher AULPC value (80.31) in JJJDi-1 isolate of *Diaporthe* spp. indicated higher virulence. In case of *Botryosphaeria* isolates, highest lesion length was observed in KSJBo-1 (10.50 cm) through wound inoculation method followed by GRPuMBo-3 (8.70 cm) which were statistically different at 21 DAI. Similarly, KSJBo-1 of *Botryosphaeria* spp. produced higher AULPC value of 74.10 followed by GRPuMBo-3 (62.10) and RKBBo-1 (47.21) which was statistically significant. The optimized inoculation techniques successfully mimicked natural infection processes, resulting in uniform and distinct symptom expression for both diseases within a predictable time frame.

Keywords : *Botryosphaeria* spp.,cashew, *Diaporthe* spp., gummosis, inoculation methods,

INTRODUCTION

Cashew (*Anacardium occidentale* L.) is one of the most valuable plantation crops of the tropical and subtropical regions, playing a pivotal role in agricultural economies through export earnings, employment generation, and livelihood security of small and marginal farmers.

The Cerrados of central Brazil is the probable centre of origin of cashew from where it spread to Europe, East Africa and India (Mitchell and Mori, 1987). In India it was introduced by Portuguese for the purpose of soil conservation in coastal Goa from where it spread to other parts of the country (Johnson, 1973). Among several *Anacardium* species in the Family Anacardiaceae, *A. occidentale* L. is an economically important plantation crop. Maharashtra, Karnataka, Tamil Nadu, Andhra Pradesh, Odisha and West Bengal are the leading cashew growing states in India. The cashew tree is a medium sized woody, evergreen, dicotyledonous tropical plant, typically attains a height of 5-8 metres, and may grow up to as much as 15m height, having width of 20m and canopy spreads up to 12m under favorable conditions. The tree has thick, resinous, spherical, scaly bark and sturdy branches.This cross-pollinated plant starts flowering " from the seedling stage, ie. " four years of age whereas grafted one produces

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flower at the age of two years. Cashew is an andromonoecious plant with both staminate and hermaphrodite flowers which occurs in the same inflorescence. Flowering lasts for 2 to 3 months. It takes 50 to 70 days to mature after pollination. Mature cashew tree has well-developed taproot system with extensive network of laterals.

Its adaptability to marginal soils and diverse agro-climatic conditions has facilitated its widespread cultivation; but several factors like climate change, intensive crop cultivation in a specific location, emerging plant diseases are growing on a novel host plant and/or in a new geography where diseases were not existent. (Gladieux *et al*. 2011; Silva *et al*.2012). The important cashew diseases in West Bengal are gummosis (Patsa 2021), shoot dieback (Patsa *et al.* 2024), Pestalotiopsis leaf spot (Patsa *et al*. 2023a), anthracnose (Jash *et al*. 2018), wilt(Patsa *et al.* 2023b; Pati *et al.* 2025) etc. Among the economically important diseases of cashew, shoot dieback caused by *Diaporthe* spp. and gummosis associated with *Botryosphaeria* spp. are recognized as particularly destructive disease and found throughout the year in the orchard . These two diseases are mainly associated with woody parts of the plants. “mainly restricted to main trunk, primary and secondary branches, etc.” Shoot dieback is characterized by progressive drying of shoots, necrosis of twigs, and eventual decline of the canopy, while gummosis manifests as gum exudation from the stem and branches, often accompanied by bark cracking and internal tissue degradation. These diseases not only reduce flowering and nut yield but also predispose trees to secondary infections, ultimately shortening the productive lifespan of the orchards.

Moreover, both the pathogens are known to establish latent infections, making early detection and accurate assessment of disease responses difficult under natural field conditions. In this context, the absence of standardized, reproducible and efficient inoculation techniques has been a major limitation in studying disease development, pathogenicity and host resistance in cashew.Isolation of pathogen from diseased woody tissue is always challenging task as there is risk of contamination from saprophyte grown on bark. Screening of different germplasm or varieties against gummosis disease in orchard to identify the sources of resistance is expensive and time consuming. The development of a method that allows early identification of sources of resistance in cashew genotypes is necessary to reduce the time and costs of these studies. The several methods i.e. injection of spore suspension, the deposition of propagules of the pathogen in cuts made on the stem and the introduction of wooden sticks colonized by the pathogen in the tissues commonly used to inoculate pathogens on the stem of woody plants in different host plant (Khanzada *et al*. 2004a,b). In jute leaf floating method, leaf swab method, floating disc method, leaf tip cut and swab method as well as stem inoculation methods were tested against stem rot of jute incited by *Macrophomina phaseolina* (Tassi) Goid to find the method which is easy to handle, less time consuming and give accurate results (Mandal *et al*. 2000; De and Mandal, 2012; De, 2016). The objective of this work was to develop a suitable and efficient method of inoculating cashew genotypes in 4-month-old seedling under nursery condition against cashew shoot dieback and gummosis. In view of these challenges, the present investigation aims to develop and standardize inoculation techniques for evaluating the response of cashew against shoot dieback caused by *Diaporthe* spp. and gummosis caused by *Botryosphaeria* spp. The outcomes of this study are expected to provide a robust experimental framework for disease assessment, contributing to a deeper understanding of cashew–pathogen interactions, and support the development of sustainable disease management and resistance breeding programs in cashew cultivation.

MATERIALS AND METHOD

Three stem inoculation methods i.e. wound inoculation; stem injection and tooth pick inoculation method were tested to search the best inoculation techniques for cashew shoot dieback and gummosis diseases associated with stem. Ten isolates collected from five districts comprising two genera *Diaporthe* spp., pathogen of shoot dieback, and gummosis caused by *Botryosphaeria* spp.was selected for the study along with control. Each isolates replicated thrice.

The test plant seedlings, cv. Jhargram-1, were 4-month-old, 50 to 60cm tall and maintained in an artificial growth chamber under artificial light with 10 h light and 14 h dark cycle at 28±1 °C temperature and 80-85 % relative humidity (RH). The upward, downward lesion length from the point of inoculation was measured individually at 6, 9, 12, 15, 18 and 21 days after inoculation (DAI). Area under lesion progress curve (AULPC) has also been calculated from the lesion data for each isolate.

Wound inoculation method

The epidermis of the stem of cashew seedling was disinfected with 70 % ethanol, washed with sterile distilled water and left to dry. One wound of 3mm diameter was made by needle at an angle of 45º to the stem 10 cm below from the tip of seedling as per protocol of Cardoso *et al*. (2010). A mycelial plug of 3mm in diameter taken from the margin of an actively growing colony on PDA of 5 days old was placed onto each stem wound. The inoculated wound was wrapped with a strip of Parafilm to prevent desiccation and contamination. Similar wound was created in control plants and inoculation with sterile PDA plug without pathogen and wrapped with parafilm.

Stem injection method

Stem was punctured to a depth of 3 mm at internodes with a disposable syringe containing suspension of pathogen (Cardoso *et al*. 2010). Needle was inserted into stem at an angle of 45º to the stem 10 cm below from the tip of the seedling. The inoculated puncture was wrapped with a strip of Parafilm to prevent desiccation and contamination. In control plant, inoculation was done with a disposable syringe containing sterile distilled water and wrapped with parafilm.

Toothpick method

In this case, inoculum was prepared on round wooden toothpicks as per protocol of Pryor *et al*. (2000). Bunch of toothpicks were bound together in a bundle with a rubber band, and each bundle was placed vertically into a small jar. 25ml of Potato dextrose broth was poured over the top of each bundle. The bundle was rolled several times inside the jar to insure uniform wetting, and the jars were autoclaved for 20 min. After cooling, 2 ml of mycelial suspension of pathogen was pipetted over the top of each bundle. The inoculated toothpicks were incubated for 5 days at 28 ±1°C in the dark. For inoculations, only the top end of the toothpick was used. One toothpick was inserted into stem of cashew seedlings at angle of 45º after surface sterilization with 70% alcohol. In control plant, inoculation was done with toothpick without pathogen and wrapped with parafilm.

RESULTS AND DISCUSSION

Three inoculation methods i.e. wound inoculation, stem injection and tooth pick method were tested to study the pathogenicity of the pathogens isolated from the stem region for cashew diseases associated with stem (Fig. 1).Ten isolates from five districts comprising two genera were selected to search the best method of inoculation along with control. Brown lesion expanding up and downwards was seen on cashew seedling inoculated through different methods with pathogen whereas in control there was only a restricted small mechanical injury.” Small lesion produced by *Diaporthe* spp., causal agent of shoot dieback of cashew, through wound inoculation method at 6 DAI (1.208cm) and it

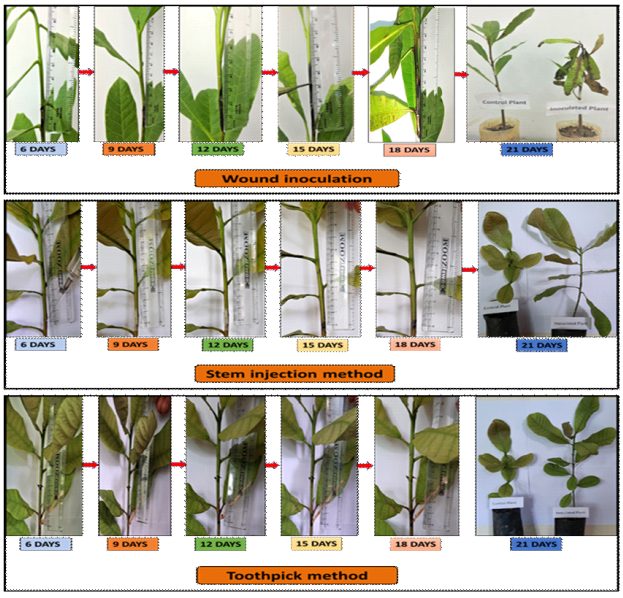

Fig 1: Lesions produced by different inoculation methods on cashew seedlings

Table1: Effect of wound inoculation method on lesion length and AULPC on cashew seedlings

Pathogen & isolate	Lesion length (cm)						AULPC
Diaporthe spp	6 days	9 days	12 days	15 days	18 days	21 days	
JJJDi-1	1.83(1.37*)	2.33(1.52)	3.30(1.81)	6.00(2.45)	8.43(2.9)	11.60(3.41)	80.31
BMJDi-1	1.30(1.16)	1.83(1.35)	2.70(1.64)	4.20(2.05)	6.60(2.57)	6.88(2.61)	58.24
DJJDi-1	1.73(1.33)	2.13(1.46)	3.00(1.73)	4.60(2.14)	5.40(2.3)	7.37(2.71)	59.01
MFBDi-1	0.00(0.22)	1.20(1.09)	1.80(1.34)	3.00(1.73)	4.55(2.13)	5.70(2.39)	40.20
KKPDi-1	1.18(1.11)	1.60(1.26)	2.28(1.51)	4.00(2.00)	5.90(2.43)	7.73(2.73)	54.68
S.Em (±)	0.04	0.05	0.05	0.04	0.09	0.16	2.30
CD (p = 0.05)	0.12	0.14	0.15	0.13	0.26	0.48	6.90
Botryosphaeria spp.							
KSJBo-1	1.70(1.32)	2.15(1.46)	3.15(1.77)	5.48(2.34)	7.83(2.80)	10.50(3.24)	74.10
GRPuMBo-3	1.35(1.18)	1.98(1.4)	2.83(1.68)	4.20(2.05)	6.68(2.58)	8.70(2.95)	62.10
RKBBo-1	0.00(0.22)	1.13(1.06)	2.15(1.46)	3.95(1.99)	5.20(2.28)	6.63(2.57)	47.21
LGPaMBo-2	0.45(0.66)	1.33(1.15)	1.95(1.39)	3.15(1.77)	5.00(2.23)	6.05(2.46)	44.03
KKPBo-1	0.00(0.22)	1.10(1.04)	2.10(1.45)	3.05(1.74)	4.85(2.20)	6.75(2.60)	43.43
S.Em (±)	0.08	0.06	0.05	0.04	0.04	0.03	1.90
CD (p = 0.05)	0.23	0.19	0.15	0.12	0.12	0.09	5.70

*Figure in parenthesis indicates square root transformed value

expanded with time up to 7.856 cm at 21 DAI whereas, lesion expansion in stem injection method has been started only on 12 days after inoculation (0.416cm) and increased up to 0.502cm on 21 DAI(Fig.2). No significant lesion has been produced by *Diaporthe* spp. through took pick method even at 21 DAI. Similarly, when *Botryosphaeria* spp., pathogen of cashew gummosis, was inoculated it was found that 7.726 cm lesion was produced through wound inoculation method as compared to 0.242 cm in stem injection method at 21 DAI (Fig.3).

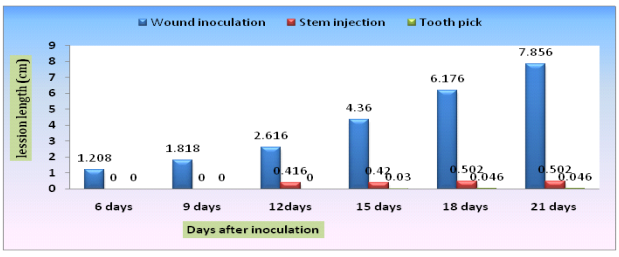

Fig 2: Lesion produced by *Diaporthe*spp. on cashew seedlings by different inoculation methods

Area under lesion progress curve (AULPC) was calculated based on lesion size produced at multiple time periods in three inoculation methods by these pathogens and it was observed that highest AULPCvalue (58.488) produced by *Diaporthe* spp. in wound inoculation method as compared to stem injection (4.756) and tooth pick inoculation methods (0.294), irrespective of isolates (Fig. 4).However, *Botryosphaeria* spp. produced comparatively maximum AULPC value (54.174) through wound inoculation method. So, wound inoculation method showed highest area under lesion progress curve value in both genera,*Diaporthe,* spp. and *Botryosphaeria* spp. as compared to others two methods.

When lesion produced by individual isolate was studied, JJJDi-1 isolate of *Diaporthe*spp. produced 11.60 cm long lesion (Table 1)in wound inoculation method at 21DAI followed by KKPDi-1 (7.73cm), DJJDi-1 (7.37cm) and BMJDi-1 (6.88cm). When area under lesion progress

Table 2: Effect of stem injection method on lesion length and AULPC on cashew seedlings

Pathogen & isolate	Lesion length (cm)						AULPC
Diaporthe spp JJJDi-1	6 days	9 days	12 days	15 days	18 days	21 days	
	0.00(0.22*)	0.00(0.22)	0.75(0.89)	0.80(0.92)	0.98(1.01)	0.98(1.01)	9.04
BMJDi-1	0.00(0.22)	0.00(0.22)	0.55(0.77)	0.50(0.74)	0.50(0.74)	0.50(0.74)	5.40
DJJDi-1	0.00(0.22)	0.00(0.22)	0.53(0.76)	0.55(0.77)	0.68(0.85)	0.68(0.85)	6.26
MFBDi-1	0.00(0.22)	0.00(0.22)	0.00(0.22)	0.00(0.22)	0.00(0.22)	0.00(0.22)	0.00
KKPDi-1	0.00(0.22)	0.00(0.22)	0.25(0.47)	0.25(0.47)	0.35(0.54)	0.35(0.54)	3.08
S.Em (±)	NS	NS	0.08	0.08	0.09	0.09	0.61
CD (p = 0.05)	NS	NS	0.25	0.24	0.27	0.27	1.86
Botryosphaeria spp.							
KSJBo-1	0.00(0.22)	0.00(0.22)	0.38(0.65)	0.48(0.72)	0.55(0.77)	0.58(0.79)	5.06
GRPuMBo-3	0.00(0.22)	0.00(0.22)	0.18(0.45)	0.35(0.63)	0.40(0.66)	0.43(0.68)	3.41
RKBBo-1	0.00(0.22)	0.00(0.22)	0.00(0.22)	0.20(0.48)	0.20(0.48)	0.20(0.48)	1.50
LGPaMBo-2	0.00(0.22)	0.00(0.22)	0.00(0.22)	0.00(0.22)	0.00(0.22)	0.00(0.22)	0.00
KKPBo-1	0.00(0.22)	0.00(0.22)	0.00(0.22)	0.00(0.22)	0.00(0.22)	0.00(0.22)	0.00
S.Em (±)	NS	NS	0.04	0.05	0.05	0.06	0.47
CD (p = 0.05)	NS	NS	0.12	0.14	0.16	0.17	1.43

*Figure in parenthesis indicates square root transformed value; NS: not significant

curve (AULPC) were calculated from lesion data of six different observation dates, it was found that same trend of results. Higher AULPC value (80.31) in JJJDi-1 isolate of *Diaporthe*spp. indicated higher virulence. In case *Botryosphaeria* isolates (Table 1), highest lesion length was observed in KSJBo-1 (10.50 cm) followed by GRPuMBo-3 (8.70 cm) which were statistically different at 21 DAI. Similarly, KSJBo-1 of *Botryosphaeria* sp. produced higher AULPC value of 74.10 followed by GRPuMBo-3 (62.10) and RKBBo-1 (47.21) which were statistically significant. When stem injection method was considered, it was found that highest lesion size of only 0.98 cm and 0.58 cm produced in isolate of JJJDi-1 and KSJBo-1, respectively (Table 2). Three isolates did not produce any lesion in stem

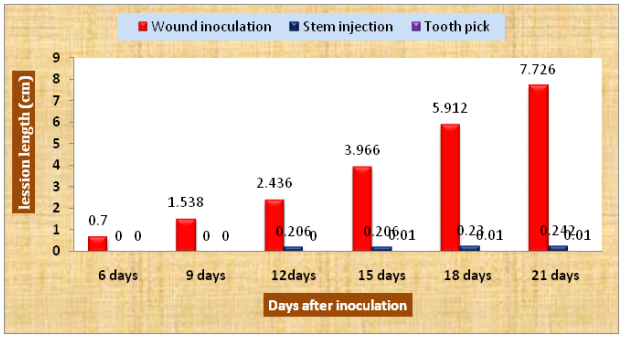

Fig 3: Lesion produced by *Botryosphaeria* spp. on cashew seedlings by different inoculation methods

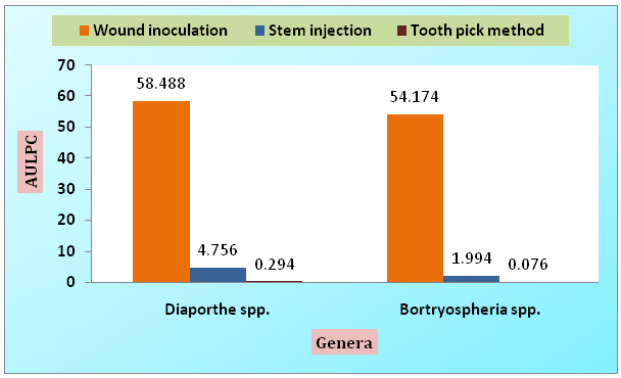

Fig 4: Comparison of different inoculation methods in terms of area under lesion progress curve

injection method. Most of the isolates produced comparatively smaller lesion on inoculation in stem injection method as compared to wound inoculation method. There was no such clearly visible lesion development on inoculated stem by toothpick inoculation method.

Standardization of inoculation method was done in order to define a best method for the early selection of germplasm resistant to gummosis and other phytopathological experiments related to host parasite interaction by testing three methodologies like wound inoculation, stem injection and tooth pick method for pathogen inoculation in susceptible variety. This experiment was practically highly valuable because it was very difficult to inoculate hard wood stem like cashew. Cardoso *et al*. (2010) tested three inoculation method for cashew gummosis and found that the bevel and toothpick methods induced darkening of the tissues around the inoculation site in the susceptible clone (CP 76) as early as 11 days after inoculation, with the sudden death of some plants in the toothpick method.

On the contrary, in the resistant clone, BRS 226, no external symptoms were appeared on cashew seedling when it was inoculated by the bevel and injection methods. In our present experiment upward and downward progression of black lesion in susceptible variety, Jhargram-1 was observed after 6 days of inoculation by wound inoculation method. Cardoso *et al*. (2010) also reported that the necrosis of the internal tissues in the plants inoculated by the injection method shows the success of the fungus infection. In our experiment toothpick method did not show any promising results because this method has a practical inconvenience due to its difficulty in penetrating the woody stem of the seedlings. This method has been used efficiently in more tender plants such as passion fruit and carrot (Pryor *et al*. 2000). The pressure exerted during the inoculation may have caused an injury to the plant tissue, causing greater stress and consequently, greater severity of symptoms.

CONCLUSION

Wound inoculation method is the easy and efficient way for studying pathogenicity and germplasm reaction to shoot dieback and gummosis diseases associated with stem in cashew.

DECLARATION

Conflict of Interest. Authors declare no conflict of interest.

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