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Research Article

Trichoderma spp. intervened activation of defensive enzymes in *Musa paradisiaca* cv. Malnad Rasbale plantlets

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ABSTRACT: *Fusarium oxysporium* f. sp. *cubense* (Foc) race is an archetypally soil-born fungus causing chief fiscal damage to farmers growing banana plants. Supervising Foc is attained by the habit of chemical fungicides which undesirably harm the soil fertility. Our investigations intended to activate the aptitude of *Trichoderma* strains for activation of PGPR and IIR. The activation and accretion of defence enzymes such as Polyphenol Oxidase (PPO), Phenylalanine Ammonia-Lyase (PAL) and Peroxidase (PO) are been amplified by inoculation, and treatment with a combination of *Trichoderma harzanium* and *Trichoderma viride* strains (T3-H1+V1). Besides combination-based inoculum treatments gave best results than individual and fungicide-treated plantlets for supervising Foc pathogenesis. Based on the results we conclude that usage of T1-H1 and T2-H2 treatments with a combination such as T3-H1+V1 gave promising results and can be used as a prominent biocontrol formulation for inducing defence enzymes and PGPR in *Musa paradisica cv.* Malnad Rasbale.

KEYWORDS: Antagonism, biocontrol, *Fusarium* wilt, *Musa paradisiaca*, *Trichoderma*

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INTRODUCTION

The global ruin of numerous banana cultivars has been illustrious by many kinds of diseases. Panama wilt initiated by *Fusarium oxysporium* f. sp. *cubense* (Foc) race 4 is dominant among them. This pathogen has been notified as a perilous disease in banana farmyards of many subtropical and tropical countries such as Australia, Malaysia, Oman, Africa, and India. (Ploetz, 2006; Ploetz *et al*., 2015). From the first day of its encounter, it has been investigated that it couldn't be controlled excellently. Biocontrol tactics for pathogenesis to plants comprise the favourable microbes which boost the PGPR (Plant Growth Promotion schedules), disease inhibition actions of soil-born pathogens, and establishment of the immune system of the plant. (Mendes *et al.,* 2011; Walters *et al*., 2013). Through biocontrol approaches, some species of *Trichoderma* have been anticipated to resist this pandemic on crops (Mohammed *et al.,* 2011; Al-Ani, 2017). Reports through several investigations suggest that the Foc pathogen infecting banana can be suppressed excellently by *Trichoderma* spp. (Calvet *et al.,* 1990; Kidane and Laing, 2008). The activity of biocontrol concerning *Trichoderma* spp. could be explained by the action of cell wall degrading enzymes, and antibiosis. (Benítez *et al*., 2004; Al-Ani, 2018). With the production of volatile compounds *Trichoderma* spp. acquired the ability to inhibit the growth of fungal pathogens (Raza *et al.,* 2013).

Trichoderma spp. is soil-born fungi belonging to the *Hypocreaceae* family. It can be used as a bio-control agent against vital plant pathogens due to its virulent and symbiotic nature. These species are ranged worldwide and are versatile in agronomy. Potentiality these fungi release many fungi toxic stuff to control pathogenesis and the induction of multi enzymes which can degrade the pathogen's cell wall material making them a unique biocontrol agent (Puyam, 2016). *Trichoderma* spp. colonizes in rhizosphere regions of banana roots, they directly impact progress and nutrition augmenting activities. Further, they nourish the defence mechanism of plants, interpreting the plant to stand against pathogens (Romera *et al*., 2019).

Current surveys concerning *Trichoderma* spp. have exposed that this cosmopolitan will colonize in the rhizospheric and root parts, interacting with an exterior layer of roots and epidermis. This mutualism will adequately define the plant from contagious mediators (Mukherjee *et al.,* 2018; Galletti *et al*., 2020; Puyam, 2016). Here when the

Trichoderma spp. starts correlating from the root part with the plant, persuades of gene expression motivated defence mechanism, plant, and root improvement (Mayo *et al*., 2016; Manganiello *et al*., 2018; Pimentel *et al.,* 2020). Somehow root-associated mutual activities of *Trichoderma* spp. for antagonistic action against Foc have not yet been stated in *Musa paradisica* cv. Malnad Rasbale, an endemic cultivar of the Malnad regions of Karnataka, India in *in-vitro* and *in-vivo* conditions. The cultivar Malnad Rasabale (silk AAB group) is pervasive in the evergreen region of the Western Ghats of Karnataka, India which is popularly known as the Malnad region. A Survey of the literature indicated that standardization of micropropagation protocol was not investigated so far on this cultivar, so in our recent study we have reported the standardized protocol for micropropagation and in the present investigation disease-resistant clones have been regenerated which has not been reported so far. This cultivar is highly liable to Panama wilt disease caused by the pathogen *Fusarium Oxysporum* f.sp. Cubense (FOC) and the farmers are stressed to get disease-free healthy planting materials

Ethyl Methanesulfonate (EMS) (Omar *et al.,* 1989; Bhagwat, 1998) a chemical mutagen and *Fusarium* culture filtrate has been used to regenerate and develop Foc tolerant banana cultivars have been reported (Matsumoto *et al*., 1995; Svabova and Lebeda, 2005; De Ascensio and Dubery, 2000). Usage of different biochemical enzymes (Thakker *et al.,* 2007; Kavino M *et al.,* 2009; Wu *et al.,* 2010) and gene markers are stated as the key proof that banana plants are tolerant to pathogenesis at the initial stage of development (Van den Berg *et al.,* 2007; Fadden *et al*., 2001; Dowd *et al*., 2004). Here an attempt has been made to develop the Foc tolerant *Musa paradisica* cv. Malnad Rasbale plantlets, which have not yet been reported by developing the *Trichoderma* spp., Foc, EMS, and fungicide intervened activation of defensive enzymes in *in-vitro* developed banana plantlets.

MATERIALS AND METHODS

Isolation of fungal samples

Trichoderma harzianum, *Trichoderma viride,* and *Fusarium Oxysporium* f.sp. *cubence* (Foc) were isolated from soil samples of Panama disease infected farmyards of *Musa paradisiaca* cv. Malnad Rasbale in and around Malnad regions of Karnataka, India. With the assistance of the serial dilution technique, pure cultures were isolated and grown on a Potato Dextrose Agar (PDA) medium (Sarangi *et al*., 2021; Ren *et al*., 2021). The pure culture was yet again grown on PDB (Potato Dextrose Broth) medium and the fungal mats were taken out individually. Individual fungal mats were crushed by pouring liquid nitrogen and crushed using a pestle and mortar. For further use, it was stored in glycerol stock at -20 °C. (Bansal *et al*., 2021)

Antagonistic effect of *Trichoderma* spp. against Foc

The antagonistic effect of *Trichoderma* spp. against the pathogen *Fusarium oxysporum* f. sp. *cubense* has been experimented with via the dual culture method (Dennis and Webster, 1971). Around 10 *Trichoderma spp.* isolates and two reference strains *Trichoderma harzianum* (NFCCI-3464) and *Trichoderma viride (*NFCCI-2552) procured from NFCCI-Agarkar Research Institute, Pune were subjected to primary screening for the antagonistic effect against FOC. During screening here *Trichoderma* spp. individually were streaked in front of the pathogen inside Petri plates and observed the antagonistic action of *Trichoderma* spp. against Foc day by day. Further, two *Trichoderma* strains that showed the highest antagonistic activity against pathogen Foc were used for PGPR following the standard protocols in laboratory conditions. (Savani *et al.,* 2020)

Fungi-Derived selective media

Pure culture of Foc, *Trichoderma harzianum,* and *Trichoderma viride* cultured on PDB and were shaken very well with the help of a rotary shaker at 80-120 rpm for 15 days. With the help of Whatman filter paper and funnel (20-25 µm), the PDB containing fungal cultures were filtered and centrifuged at 5000 rpm for 15 min. A membrane filter with 0.22 µm pore size helped to remove all cell debris of the supernatant obtained. Obtained fungal filtrates were augmented aseptically to autoclaved and cooled MS media (MS+ 5 mg/L BAP + 5-25% (v/v) fungal culture filtrates) and used as selection media concerning treatments. (Venkatesh *et al.,* 2013)

Mutagen treatment

In-vitro developed *Musa paradisiaca* cv. Malnad Rasbale shooting stage plantlets were treated with an aqueous solution of Ethyl Methane Sulfonate (EMS) with a range of 0.1-0.5% respectively. The plantlets were disturbed with the help of a gyratory shaker (100 rpm) for one hour at 28±2 °C. By fine wash of sterile distilled and autoclaved water, plantlets were relocated to restoration media (MS media +5 mg/L BAP+ 2% activated charcoal) and cultured for the 30th day (Venkatesh *et al.* 2013). Well-developed regenerants with roots and shoots were transferred to playhouse conditions. *In-vitro* regenarents bearing leaves and plantlets developed in the pot to a length of 30-35 cm and bearing 4-5 leaves in the poly house were isolated to screen the disease-resistant banana plantlets by using biochemical assays against Foc (Venkatesh *et al*. 2013).

Chemical fungicide treatment

Preparation for fungicide treatment inoculum was achieved, and commercially available Carbendazim ($C_9H_9N_3O_2$) powder was mixed with 2-10 g in 100 ml of

Table 1. Treatment combinations for banana plants in in-vitro conditions are as follows

Sl. No.	Treatments	Fungal filtrate, mutagen and fungicide treatments
1	T1-H1	*Trichoderma harzianum*
2	T2-V1	*Trichoderma viride*
3	T3-H1+V1	*Trichoderma harzianum* + *Trichoderma viride*
4	T4-F1	*Fusarium oxysporum* f.sp. *cubense* (Foc)
5	T5-H1+V1+F1	*Trichoderma harzianum* + *Trichoderma viride* + Foc
6	T6-E1	EMS (Ethyl methane Sulfonate)
7	T7-E1+F1	EMS+ Foc
8	T8-C1	Carbendazim ($C_9H_9N_3O_2$)
9	T9-C1+F1	Carbendazim+Foc

distilled H_2O respectively. Later the prepared chemical fungicide inoculum was poured into autoclaved MS media and left to cool. *In-vitro*-developed banana plantlets were cultured in MS media (MS+ 5 mg/L BAP + 2-10% Carbendazim). Later the changes in the inoculated plantlets were observed up to the 30th day. (Jarwar *et al.,* 2021)

Collection of plant samples

Pseudo-stem derived, well-developed disease-free *Musa paradisiaca* cv. Malnad Rasbale plants developed in *in-vitro* conditions were used for inoculum treatment. 25-30 days old *in-vitro*-developed greenhouse developed plants were used for pot experiment studies. 28 °C of temperature was maintained under *in-vitro* conditions and 28-30 °C under greenhouse conditions. Fungal isolates with the highest antagonistic activity were selected for the treatment of plants in laboratory and greenhouse conditions. For each treatment of fungi, mutagen and fungicide, triplicates (3*10) were used in *in-vitro* conditions by varying the concentration with a range as mentioned before. When compared to *in-vivo* conditions in the pot experiment, individual treatment was given by pouring the prepared inoculum at the specific concentration to MS media in *in-vitro* conditions and results were noted up to the 30th day.

Pathogenicity test

Root dipping method

With aseptic conditions under a laminar airflow hood *in-vitro*-derived banana plantlets were injured with the help of a scalpel and immersed in pathogen suspension culture (10^6 spores/mL) for 15 min. Plants developed with roots were aseptically transferred to MS media fortified with 0.25 activated charcoal. Control plants were just washed with sterile H2O and transferred to rooting media as mentioned before (Tripathi *et al*., 2008).

Pseudostem injecting method

With the help of a sterile syringe under laminar airflow hood suspension culture of the pathogen (100 µL; 10^6 spores/mL) was injected into the middle part of the plantlet. With concern to 16 hrs, photoperiod plantlets were transferred to rooting media and maintained at 28±2 °C. For spore suspension, culture (10^6 spores/mL) was directly inoculated to *in-vivo* potted plantlets. (Venkatesh *et al.* 2013). For scrutiny of disease inhibition activity in *in-vitro* conditions, *Trichoderma* spp., EMS, Foc, and fungicide culture filtrates derived plants were isolated aseptically and pathogenic efficacies of Foc on these selected clones were evaluated by using biochemical assays by taking leaves of *in-vitro* developed plantlets in greenhouse conditions.

Biochemical assays for screening of disease resistance banana plantlets

To investigate the activities of the enzymatic and biochemical compounds after the plants have been developed by media fortified with fungal filtrates fresh leaf samples of *in-vitro* regenerants of *Musa paradisiaca* cv. Malnad Rasbale was taken for the preparation of crude enzyme extract for the evaluation of Peroxidase (PO) (Savani *et al*., 2020), Polyphenol Oxidase (PPO) (Mayer *et al.,* 1966), and Phenylalanine Ammonia-Lyase (PAL) (Dickerson *et al.,* 1984) activities.

Statistical analysis

Here the investigation of enzymatic activities in plantlets was examined when the plants were developed in greenhouse conditions and were ready for *in-vivo* planting. Obtained records were examined using ANOVA statistical software.

RESULTS AND DISCUSSION

Antagonistic effect of *Trichoderma* spp. against Foc

An observation regarding the antagonistic influence of *Trichoderma* strains significantly reduced the growth of Foc. Out of 10 *Trichoderma* strains and 2 reference strains, only 2 remote, screened strains bared the premier antagonistic activity against Foc and were selected for promoted studies. Nominated fungal strains were scrutinized for PGPR activities. It was notified that the selected 2 strains persuaded the PGPR activities. Fig. 1A. represents the concentration of fungal filtrates in MS media. Based on fungal strains treated outcomes enzymatic studies were carried out on the 0, 30th, and 45th days of treatments (Fig. 2A-2C). These included *Trichoderma harzanium* (T1-H1) and *Trichoderma viride*

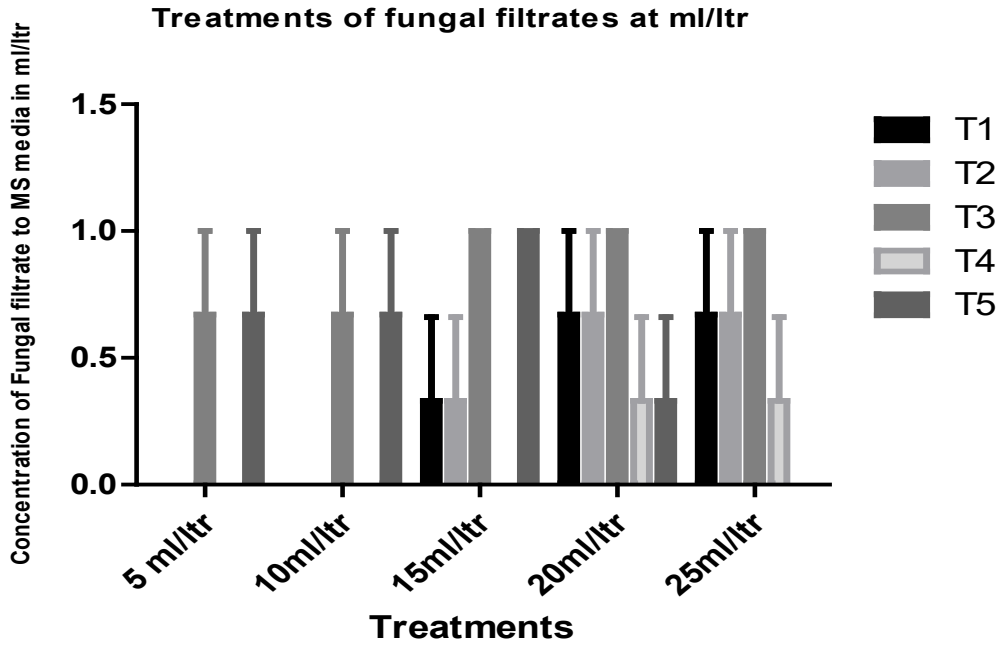

Fig. 1A. Representation of treatments of fungal filtrates at ml/ltr.
Values are represented in mean ±S.E.

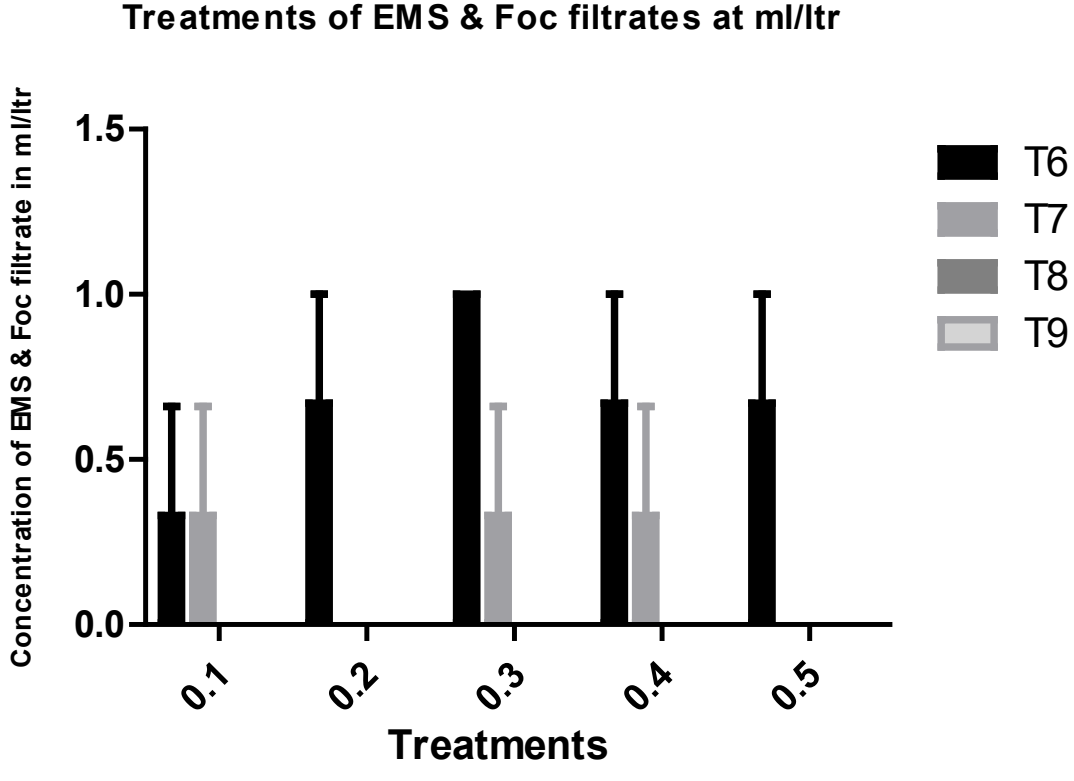

Fig. 1B. Representation of treatments of EMS and Foc filtrates at ml/ltr.
Values are represented in mean ±S.E.

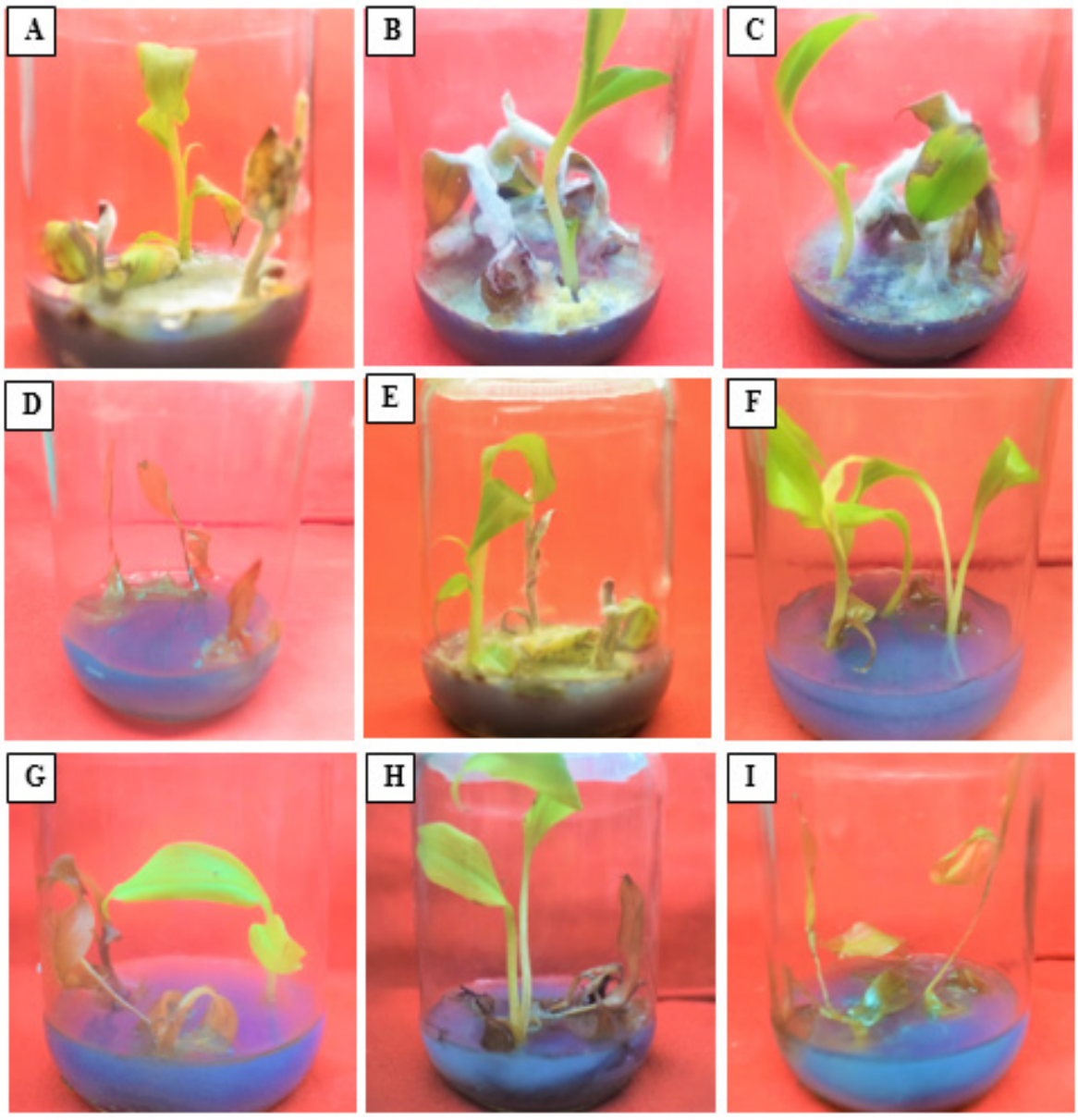

Fig. 2A-2I Represents different growth parameters grown in the fungal filtrate, mutagen, and fungicide treatments.

(T2-V1). A combination of 2 *Trichoderma* strains and Foc strain (T5- H1+V1+F1) was also treated and evaluated.

Mutagen and fungicide treated *in-vitro* regenerants

EMS mutagen (T6-E1), (T7-E1+F1), Carbendazim ($C_9H_9N_3O_2$) (T8- C1), and T9 treated *in-vitro* developed *Musa paradisiaca* cv. Malnad Rasbale plantlets leave aged 0, 30th day of development and polyhouse transferred 15th-day plantlets leaves were taken for enzymatic studies. Fig.1B. Represents the concentration of EMS, Foc, and fungicide used for treatments and the percentage of plants that survived from the day one treatment. Trial leaves were taken separately according to the treatments.

Development of defense-related enzymes by *Trichoderma* strains

Contemporary investigations disclosed that a developed range of defence enzyme activity was notified, such as PPO, POX, and PAL, and was recorded. Investigated results showed that individual treatment of *Trichoderma* strains didn't develop defence enzymes but combination-based treatment (T3-H1+V1) *Trichoderma harzianum* + *Trichoderma viride* showed the best results in increasing defence triggering activities (Fig. 2C). But EMS-treated plants also showed development in enzymatic activity (Fig. 2F and 2G). All the treatments from T1-T9 were inoculated

individually for triplicate samples of plantlets in *in-vitro* conditions but treated individually at poly house conditions and were experimented with for enzymatic developmental calculations.

1. *Trichoderma harzanium* treated triplicate plantlets, (T1-H1) (Fig. 2A) 2. *Trichoderma viride* treated plantlets. (T2-V1) (Fig. 2B). 3. *Trichoderma harzianum* + *Trichoderma viride* combination mediated media showed PGPR and IIP (T3-H1+V1) (Fig. 2C). 4. *Fusarium oxysporum* f.sp. *cubense* (Foc) treated plantlets showed necrosis after a few days of inoculation, but some of them survived. (T4- F1) (Fig. 2D). 5. *Trichoderma harzianum* + *Trichoderma viride* + Foc mediated media, here also plant showed resistance to pathogenesis. (T5- H1+V1+F1) (Fig. 2E). 6. Triplicate *Musa Paradisica* cv. Malnad Rasbale plantlets treated with EMS (Ethyl methane Sulfonate) were inoculated. (T6-E1) (Fig. 2F). 7. Out of triplicates, only one plantlet showed resistance in EMS+Foc treatment. (T7-E1+F1) (Fig. 2G) 8. Carbendazim ($C_9H_9N_3O_2$) treated triplicates plants only one plant survived till the 30th day in *in-vitro* conditions. (T8-C1) (Fig. 2H) 9. But in Carbendazim+Foc treated triplicates with different concentrations all plants showed necrosis. (T9-C1+F1) (Fig. 2I).

Proficiency of *Trichoderma* strains in controlling Panama wilt

In the present investigation on *Musa Paradisica* cv. Malnad Rasbale artificially infected with Foc race 4 was found successfully reduction in disease inhibition activity treated with combination-based treatment *Trichoderma harzianum* + *Trichoderma viride* (T3-H1+V1) as mentioned in Fig. 1B. Procrastinating of pathogenesis was notified after treatment in *in-vitro* and playhouse conditions. In the case of *Trichoderma harzanium* and *Trichoderma viride* individually treated plantlets severity of survivance was notified (Fig 1B) *In-vitro* regenerates treated with T1-H1 and T2-V1 and T4-F1 (Fig. 2A, 2B and 2D) individually gave the lowest survivance values, where a mixture of *Trichoderma* strains (T3-H1+V1) recorded the highest rate of survivance for pathogenesis values from 0,30th and 45th day of *in-vitro* regenerated plantlets up to playhouse development. (Fig. 1B). Investigated results raised a conclusive idea that individual treatment of biocontrol

Fig. 2J-2L. Representing morphology of polyhouse grown *Musa paradisica* cv. Malnad Rasbale treated with different treatments.

Table 2. Treatment and PAL enzyme activity with different time intervals. Values are represented in mean ± S.E

PAL enzyme activity and Treatments	Time intervals		
	0 days	30 days	45 days
T1	89.23±0.50	83.93±0.73	151.63±0.72
T2	86.50±0.78	119.57±0.27	148.87±0.20
T3	90.33±0.58	400.50±0.58	420.20±0.97
T4	79.57±0.63	96.23±0.66	110.23±0.37
T5	86.20±0.56	99.80±0.23	123.43±0.77
T6	90.90±0.56	359.83±0.41	400.27±0.67
T7	88.57±0.79	103.67±0.09	120.13±0.54
T8	89.37±0.58	0	0
T9	83.93±0.73	0	0

Table 3. Treatment and PO enzyme activity with different time intervals. Values are represented in mean ± S.E

PO enzyme activity and Treatments	Time intervals		
	0 days	**30 days**	**45 days**
T1	97.90±0.23	118.47±0.65	190.03±0.47
T2	86.50±0.26	121.60±0.32	137.70±0.81
T3	86.00 ±0.49	380.53±0.67	416.53±1.03
T4	91.87±0.45	90.87 ±0.15	120.13±0.98
T5	91.53±0.73	94.80±0.58	101.33±0.55
T6	90.43±0.35	340.60±0.58	396.77±0.35
T7	86.20±0.57	98.80±0.17	110.87±0.15
T8	84.83±0.32	0	0
T9	87.50±2.50	0	0

Table 4. Treatment and PPO enzyme activity with different time intervals. Values are represented in mean ± S.E

PPO enzyme activity and Treatments	Time intervals		
	0 days	**30 days**	**45 days**
T1	100.07±0.90	125.40±0.69	192.67±0.91
T2	96.43±0.58	130.80±1.51	186.80±0.71
T3	99.70±0.38	434.73±0.88	460.27±0.53
T4	86.97±0.74	99.23±0.35	120.03±0.73
T5	82.83±0.67	94.90±0.72	99.37±0.50
T6	94.70±1.22	390.50±0.67	455.90±0.70
T7	85.07±0.49	112.63±1.02	160.43±0.50
T8	87.20±0.72	0	0
T9	89.77±0.75	0	0

strains was less effective than combination-based biocontrol treatments and reduced the pathogenesis of Panama disease in *in-vitro* and playhouse conditions.

1. Polyhouse developed T3-H1+V1 treated banana plant showing healthy shoot growth (Fig. 2J) 2. Polyhouse developed T3-H1+V1 treated banana plant showing healthy shoot growth (Fig. 2K) 3. T.S. of pseudostem treated with T3-H1+V1 showing no pathogenesis (Fig. 2L)

Repercussion of *Trichoderma* strains

Substantial elevation of several leaves, height, and pseudostem elaboration was witnessed by the management of combined *Trichoderma* strains over the treatment of Foc (Fig. 2J and K). By the treatments of T1-H1 to T9-C1+F1 excluding T3-H1+V1 revealed optimizing results but T3-H1+V1 disclosed the best results by assisting the PGPR activities but other treatments failed to possess PGPR activities (Fig 2A-C and E-I) (Fig. 1A). Plants treated with Foc separately got infected and the demise of plantlets in *in-vitro* and polyhouse conditions was observed (Fig 2D). EMS-treated plants were also well developed in *in-vitro* conditions and enzymatic activities were less compared to biocontrol-treated plantlets in experimented conditions. (T6-E1 and T7-E1+F1) (Fig. 1B)

Current investigation revealed the effect of specific and combination treatments of fungi, fungicide and mutagen treatments on activation of defence enzymes, disease severity, and disease inhibition in *Musa paradisiaca* cv. Malnad Rasbale upon inoculation with Foc. Considering all the above parameters effect of *Trichoderma* strains on

PGPR and defence-related activities was studied. The highest level of PAL, PO, and PPO activity was notified during the present investigation when the combination-based inoculum treatments (T3-H1+V1) were achieved. When compared to vegetative growth and declined pathogenesis gave rise to a response to us that Induced Systemic Resistance (ISR) retort of the plant would have stimulated, it was notified by testing the PAL activity. Combination-based treatment of fungal strains acquired the highest PAL activity while EMS-treated plants ranked the second highest PAL activity.

Investigated results by us state that combination-based and mutagen-treated plantlets in *in-vitro* conditions played a decisive role in activating Innate Immune Response (IIR). Reports by Kavino M (2009) and Singh *et al.,* (2019) also indicated that in combined treatments the values of PO, PPO, and PAL significantly increased It clearly shows that *Trichoderma* strains colonized plants at first and induced antagonistic activity against Foc for disease inhibition was observed next PGPR was succeeded. Enduring investigations in this zone will confidently lead us to more use of biocontrol agents also at the field level. Mutagen-treated plantlets also showed progressive growth but this may lead to genetic alterations in the future days, so biocontrol work has been focused on us (Encyclopedia of Food and Health, 2016). *Fusarium Oxysporium* f.sp. *cubence* race 4 is a soil tenant and through this Panama, wilt will be caused, it will persist in the soil even for 15-20 years (Lakshmanan *et al.,* 1987).

The persistent existence of Foc in the soil will be a challenging task for banana cultivating farmwards to obtain the expected yield. Besides here focus on the deliberate use of pesticides is leading to harmful effects on the environment and has led to public concern. Hence, it has a subservient to identify the antagonist which will efficiently manage the Foc wilt of the banana. In the present investigation T3-H1+V1 was more effective than EMS mutagen treatment, but not by the solo treatments of fungal samples and fungicide treatment. Similarly, many findings have been reported regarding endophytes that controlling Foc race 4 has been achieved in many crops and banana plants by the mixture of endophyte inoculum in and playhouse conditions (Fishal *et al.,* 2010; Gopalakrishnan *et al.,* 2011; Sun *et al*., 2011). Acquiring the PGPR, several actions and antibiosis can be achieved only through a mixture of fungal strains to control pathogenesis but not by individual biocontrol agents. (Crump, 1998; Kavino *et al.,* 2008).

CONCLUSION

Globally Panama disease is ruining the farmers for the production of bananas in farmyards. From its emblematic pathogenesis character in the environment, controlling this wilt has turned into devastation. As this pathogen is soil-born, it directly infects the plant's roots, pseudostem parts and enters into the vascular system, and causes necrosis to the host. The present investigation gave us a clear report that inoculates the *Trichoderma harzianum* + *Trichoderma viride* (T3-H1+V1) strains with pathogenesis-induced *Musa Paradisica* cv. Malnad Rasbale plantlets to control Foc wilt were successful. T3-H1+V1 strains were proficient in developing the maximum intensity of defence enzymes *in vitro* situations. Obtained results interrupt that the combined use of biocontrol strains assisted with the defence mechanism by inducing systemic resistance against pathogenesis. Groundwork and standardized protocol for the development of biocontrol formulation to manage Foc at field levels could be achieved by this investigation. But time management, methods of application, MST (Multi Seasonal Trials) for the conveyance of bio formulation in different climatic areas, and soil diversity are yet to be known in *in-vivo* conditions.

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