Eco-Friendly strategy to Control Soil-Borne Phytopathogens *Macrophomina phaseolina* and *Fusarium oxysporum*, Employing Synergistic PhytoMycoBiological Combination

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Abstract. Plant fungal pathogens *Macrophomina phaseolina*, *Fusarium oxysporum* are serious limitations to agricultural productivity due to their extended survival, wide host range and resistance to synthetic fungicides. Presently, market provides limited options of phytobiological agents for biocontrol of pathogens. The Manuscript proposes a sustainable PhytoMycoBiological (PMB) approach for effective pathogen control. Whole leaf extracts of *Ricinus communis*, *Calotropis procera* were tested for their pathogen growth suppression ability and were found to be 1.1 fold (*Ricinus* vs. *Fusarium*) and 1.5 fold (*Ricinus/Macophomina*; *Calotropis/Macrophomina*) fold more effective as compared to the conventional Neem (*Azadirachta indica*) extract applied at same dosage. In the dosage dependency experiments *R. communis* exhibited maximum inhibition, arresting ~ 50% growth in both pathogens where Neem could only restrict 30% - 40% growth of *M. phaseolina*, *F. oxysporum* respectively. All results were statistically analyzed (one-way ANOVA and post hoc test, Tukey HSD) and pathogen control ability found statistically significant ($p < 0.001$). Further, when these plant leaf extracts (PLE) were supplemented with equal amount of cell-free extract (CFE) from native biocontrol agent *Trichoderma harzianum* (T103), the antifungal effect was more pronounced (increased by 1.1 fold). CFE + *R. communis* combination was by far most impactful (64.35% growth reduction in *M. phaseolina* and 60% against *F. oxysporum*). The results reflect synergistic relationship between the action of phytobiologicals from PLE and CFE of T. *harzianum*, providing primary scientific evidence for developing most advantageous PhytoMycoBiological formulations as environmentally friendly substitutes for pathogen control. This combined strategy holds a lot of potential in terms of sustainable control of the devastating soil-borne infections in food production systems.

Keywords: *Ricinus communis, Calotropis procera, Trichoderma harzianum, Macrophomina phaseolina, Fusarium oxysporum,* antifungal activity, biocontrol, phytobiological extracts.

1. Introduction

Plant fungal pathogens represent a major limitation to the world agricultural output, as they result into the loss of substantial yields and poor quality of crops [1]. These include mostly, *Fusarium oxysporum* and *Macrophomina phaseolina* which are common and infect a wide range of economically valuable crops. The causal agent of charcoal rot, *M. phaseolina*, is especially widespread in semi-arid and tropical areas, and it remains in the soil in the form of resilient microsclerotia and severe root and stem rot when it is under drought stress [2,3]. Similarly, *F. oxysporum* induces vascular wilt diseases, which result in systemic colonization, blockage of the xylem and the death of the plant [3]. The fact that these pathogens survive in soil and have a wide host range makes their control difficult,

especially in intensive agricultural practices [3,4]. Traditional methods of control depend much on fungicides of a synthetic nature. Although it is effective in the short run, its repetition has resulted in development of resistant strains of pathogens, environmental pollution, and build up of toxic remnants in agricultural products [5]. These issues have heightened the effort to find sustainable and eco-friendly options that would follow integrated disease management models[5]. Plant-derived bioactive compounds and microbial biocontrol agents have become one of the candidates in this regard due to their biodegradability, multi-targeting activity, and a smaller environmental impact [4]. Medicine and non-edible plants used as botanical extracts have a variety of secondary metabolites that have antimicrobial and antifungal effects alkaloids, phenolics, flavonoids, terpenoids and saponins. *Azadirachta indica* is a proven plant which has antifungal property, antibacterial property and many more [6]. *Ricinus communis* also known as castor, contains ricin, ricinine and other phenolic compounds which have been reported to prevent fungal growth by disrupting the membrane and interfering with its metabolism. Likewise, the plant, *Calotropis procera* is highly endowed with cardenolides, proteolytic enzymes, and latex-produced bioactives which show antimicrobial potential [6]. Although their antifungal efficacies have been recorded, the individual efficacy of plant extracts could be variable with respect to concentration, sensitivity of the pathogen and stability of the formulations [7,8]. The mechanism behind the antifungal property of plant extracts. The presented multi-targeted mode of action includes the destabilization of the membrane caused by ergosterol binding and lipid peroxidation induction of oxidative-, stress-based apoptosis through the accumulation of intracellular ROS and mitochondrial dysfunction; the inhibition of cell-wall biosynthesis by means of the chitin and β-glucan synthase inhibition; enzyme and metabolic disruption leading to ATP depletion and the activation of systemic-resistance signaling, the pathways result in fungal cell death but at the same time, they stimulate plant defence mechanisms, which increased the potential of phytobiological interventions in a broad-spectrum with resistance-limiting effect [9,10,11].

The use of biological control by use of antagonistic fungi especially *Trichoderma harzianum* has received significant attention due to its complex process of pathogen suppression. *T. harzianum* has an antagonistic activity based on mycoparasitism, nutrient and space competition, antifungal metabolite production, and cell-wall-degrading enzyme secretion including chitinases, proteasese [12] . Enzyme fractions, intracellular as well as extracellular contribute to the degradation of cell walls of fungi and hence a higher pathogen suppression. Combination of enzymatic fractions and phytobiological extracts can provide synergistic effects, which can increase the antifungal effects through concomitant biochemical and enzymatic destabilization of pathogenic fungi [13]. Recent studies highlight the importance of using combinatorial biocontrol approaches where several bioactive materials are used to overcome the shortcomings of using one agent at a time. However, the comparative assessment of plant extract-enzyme complexes, especially those where *Ricinus communis* and *Calotropis procera* are used together with *T. -harzianum* enzyme fractions, is scarce. The model concentration dependent responses and synergistic interaction of these drugs is critical to maximizing the formulation performance and to achieve reproducible antifungal activity. The current paper, therefore, aims at comparatively assessing the antifungal effectiveness of *Ricinus communis*, *Calotropis procera procera* extracts and *Azadirachta indica* alone and *Ricinus communis, Calotropis procera* extracts as combined with cell free extract of *Trichoderma harzianum* against *Macrophomina phaseolina* and *Fusarium oxysporum*. This study will attempt to clarify how phytomycobiologicals as sustainable eco-friendly alternatives may be used in the management of devastating soil-based fungal diseases in agriculture by analyzing radial mycelial growth inhibition at various concentrations.

2. Materials and methods

2.1 Strains of Fungi and Conditions of Culture

Fusarium oxysporum and *Macrophomina phaseolina* were obtained in the Microbial Type Culture Collection (MTCC). Inoculation was done on fresh Potato Dextrose Agar (PDA) plates which were then left to grow at 28 ^{o}C, to ensure that the organisms grew well.

2.2 Preparation of Ethanolic Leaf Extracts

Calotropis procera, Ricinus communis and *Azadirachta indica* were used as the leaves of plants in the nearby environment, which were healthy. A sequential water washing protocol that involved the use of running tap water then sterile distilled water was applied on them to eliminate the particulate matter and the surface contaminants. Then the foilage was dried in the shade over 10-12 days until it reached a constant weight. The dried tissue was subsequently ground into a fine powder by using a sterile grinder and ethanolic extracts were produced separately through a Soxhlet apparatus. The level of extraction was continued until the solvent in the siphon tube turned clear. Rotary evaporator was used to concentrate the resulting extracts at 40^{o}C under reduced pressure. Crude extracts were kept at 4 ^{o}C to be analyzed further.

2.3 Phytochemical Characterization of plant extracts

Primary phytochemical screening of ethanolic extracts was done using traditional qualitative methods to identify major secondary metabolite groups. Saponins were detected by use of froth test, tannin by ferric chloride test, flavonoid using alkali reagent test, and alkaloid using Mayer and Wagner assays. The phytochemical classes were determined by the visible colorimetric changes or precipitation.

2.4 Pathogen Challenge Assay

Pathogen challenge experiments were performed to measure the antifungal properties of the plant extracts against the test pathogens in controlled test in vitro conditions. Three treatment concentrations were prepared based on the following solutions: T1, T2 and T3. In the case of *Ricinus communis*, the concentrations were T1 (0.14 g/ ml), T2 (0.26 g/ ml) and T3 (0.40 g/ ml) and in the case of *Calotropis procera*, the concentrations were T1 (0.13 g/ml), T2 (0.25 g/ ml) and T3 (0.38 g/ ml). 4x strength of each treatment was used in individual Petri plates to allow equal distribution and exposure to the pathogen. The concentration of T3 (0.37g/ml) was tested in the case of *Azadirachta indica* (neem), as the T3 (0.37g/ml) is well-known in previous research due to its proven phytobiological activity. Each of the treatments was carried out thrice to guarantee reliability of the experiment and statistical soundness. The R statistical software was conducted on the data in order to establish significant differences between treatments.

2.5 Trichoderma harzianum Isolation of Cell-Free Extract and Preparation of Enzyme Plant Extract Combinations

Revival of *Trichoderma harzianum* was done on a potato dextrose broth media under aseptic conditions after which the growth was re-cultured in large quantities to produce

metabolites. The culture broth was incubated and then filtrated and centrifuged to get a clear cell-free extract by eliminating fungal biomass and cell debris. The supernatant is collected which contained the metabolites and enzymatic constituents, was utilized as the cell-free extract. The cell-free extract was used in three different concentrations and then mixed with plant extracts in the same proportions (1: 1 ratio).Three treatment concentrations were prepared based on the following solutions: T1, T2 and T3. In the case of *Ricinus communis*, the concentrations were T1 (0.14 g/ ml), T2 (0.26 g/ ml) and T3 (0.40 g/ ml) and in the case of *Calotropis procera*, the concentrations were T1 (0.13 g/ml), T2 (0.25 g/ ml) and T3 (0.38 g/ ml) and 4x strength of each treatment was used in individual concentration for each.This plant extract formulation were subsequently tested on the target fungal pathogens to determine the improved antifungal activity that is caused the synergistic biochemical interactions between fungal metabolites and phytochemicals.

3. Results

3.1 Phytochemical Profile of Ethanolic Leaf Extracts

The qualitative phytochemical evaluation of the ethanolic leaf extracts of *Calotropis procera* and *Ricinus communis* species determined a range of bioactive secondary metabolites that have been already reported on antifungal activity as shown in Table 1. The positive result of the saponins in the two plant extracts is demonstrated by formation of a stable foam during the foam test implying that the saponins produced are able to disrupt the cell membrane of fungi and to trigger cellular leakage. The presence of alkaloids in all the extracts was verified using the Mayer test whereby the appearance of a yellowish-white precipitate indicated the possible effect of inhibiting the fungal enzymatic activity and metabolic processes. Flavonoids were identified with the help of the alkaline reagent, which changed the color to yellow temporarily, appearing and disappearing, which suggests that it can prevent the germination of the spores and limit the development of the hyphea. Phenolic constituents were observed in both extracts as the extracts were able to develop green to black color in the ferric chloride test, which is a good indication of their ability to induce oxidative stress in fungal cells. The Liebermann–Burchard reaction was also used to identify sterols that generated a blue-green color, indicating the participation in the regulation of fungal signaling routes and inhibition of pathogenicity. Also the presence of tannins was determined by the gelatin test that produced a white precipitate and this shows a tendency to undermine the stability of fungal cell walls thereby causing structural defects and cell lysis. Together with these phytochemical results, the strong antifungal potential of the extracts of *Calotropis procera* and *Ricinus communis* is revealed.

Table 1: Qualitative Detection of Secondary Metabolites in Plant Extracts

Phytochemicals	Test Name	Results	*Calotropis procera*	*Ricinus communis*
Saponins	Foam test	Formation of stable foam	+ +	+ +
Alkaloids	Mayer's test	Yellowish-white ppt form	+ +	+ +
Flavonoids	Alkaline Reagent test	Yellow color disappears	+ +	+ +

Phenolic Compounds	Ferric Chloride test	Green color	+ +	+ +
Steroids	Lierberm ann-Burchard test	Blue color	+ +	+ +
Tannins	Gelatin test	Formation of white ppt	+ +	+ +

Note: + + = most abundant

3.2 In Vitro Antifungal Activity of Plant Extracts

The attenuation of radial mycelial growth by both the *Macrophomina phaseolina* and *Fusarium oxysporum* in the pathogen challenge experiment was clearly concentration dependent and in comparison, the *Ricinus communis* showed a relatively high concentration dependent concentrations. Increased inhibition was observed at T1 (0.14 g m/l), T2 (0.26g/ml), and T3 (0.4g/ml) , and T3 produced the highest suppression of the growth of mycelium in both pathogens (Fig. 1). In *M. phaseolina*, using *R. communis* resulted in severe growth inhibition and observable hyphe morphology distortion and tissue damage at elevated concentrations, which is a result of direct cellular interaction. In the same way, in *F. oxysporum*, it was observed that there was a considerable growth retardation at all the concentrations tested, and the highest growth retardation was recorded at T3 (0.4 g/ml). Comparatively, the antifungal activity in the case of *Calotropis procera* was also dose-dependent across T1 (0.13 g/ml), T2 (0.25g/ml), and T3 (0.38g/ml), showing a steady decreasing radial growth as the concentration increased. In high doses, the effect of the colony diameter was to be reduced slightly and the effect of the hypha density was moderate indicating that it inhibited growth. Even though the overall intensity of the inhibition was relatively smaller than the one of the case of the *R. communis*, the extract showed a consistent and gradual antifungal activity in all treatments.

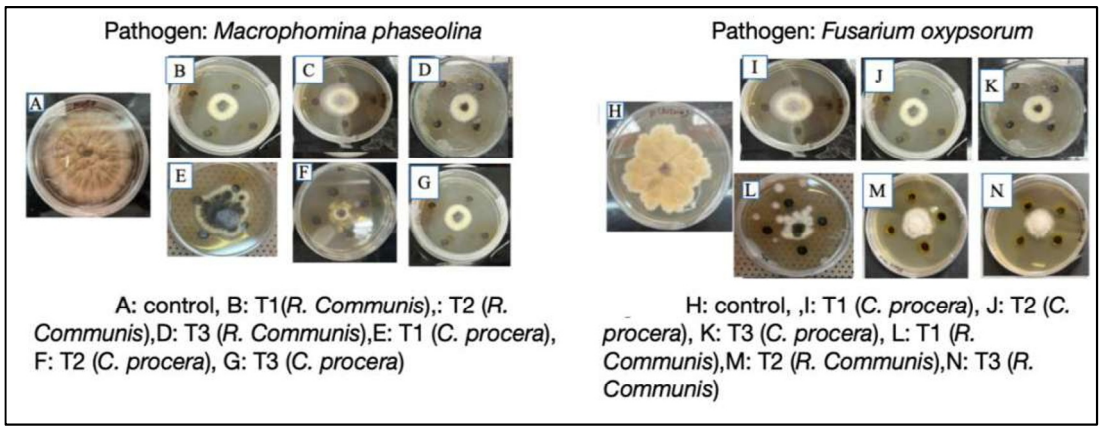

Fig. 1. Linear growth of pathogens.

3.3 Statistical Analysis of Treatment Effects on Fungal Growth

The results demonstrated a clear concentration-dependent suppression of pathogen growth across treatments (T1–T3), as illustrated in Fig. 2 (A–D), where progressive divergence between control and treated curves became increasingly evident over successive days of

incubation. In Fig. 2A, *Ricinus communis* markedly reduced the radial growth of *Fusarium oxysporum*, with treated lines (T1–T3) consistently remaining below the control trajectory and T3 exhibiting the greatest restriction of mycelial expansion, corresponding to 48% inhibition. Similarly, Fig.2B shows substantial suppression of *Macrophomina phaseolina* by *R. communis*, with a clear dose–response gradient and 44% inhibition at T3, although the magnitude of inhibition was slightly lower than that observed for *F. oxysporum*, indicating comparatively higher sensitivity. In contrast, *Calotropis procera* also displayed inhibitory activity across all treatments, but the separation between treatment and control curves was less pronounced than in *R. communis*. At T3, *C. procera* achieved 33.4% inhibition against *F. oxysporum* (Fig. 2C) and 35% against *M. phaseolina* (Fig. 2D), with *M. phaseolina* showing marginally greater susceptibility—an inverse response pattern compared to *R. communis*. Comparative evaluation further established a hierarchical efficacy pattern (*R. communis* > *Azadirachta indica* > *C. procera)*, with *Azadirachta indica* demonstrating intermediate inhibition (40% against *F. oxysporum* and 30% against *M. phaseolina* at T3). The graphical trends collectively highlight the progressive reduction in radial growth rate relative to the control, confirming biological consistency and reproducibility of treatment effects. Two-way ANOVA followed by Tukey's HSD post hoc analysis validated that all treatments significantly reduced mycelial growth compared to the control ($p < 0.001$). A strong dose-dependent response was evident in most extract pathogen combinations, particularly in the *R. communis–M. phaseolina* and *C. procera–M. phaseolina* interactions, where T3 consistently produced the highest suppression. However, a slight non-linear response was observed in the *R. communis–F. oxysporum* interaction, where T1 exhibited comparatively strong inhibition, suggesting a potential concentration-specific physiological or metabolic interaction.

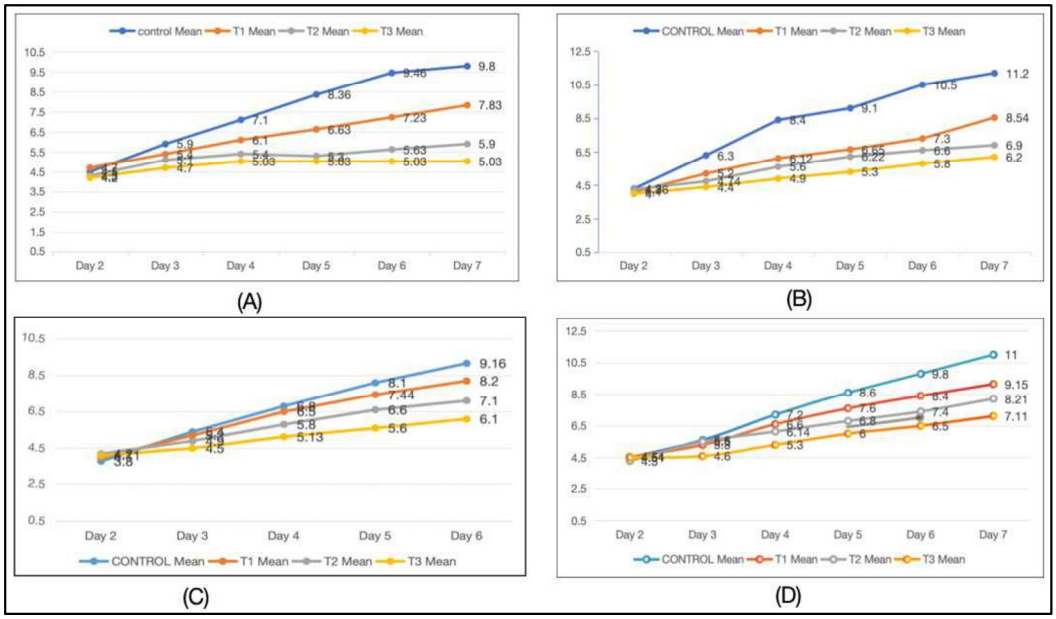

Fig. 2. Effect of Phytobiological Extracts on Radial Growth and Mycelial Inhibition of *Fusarium oxysporum* and *Macrophomina phaseolina*
(A) *Ricinus communis* against *Fusarium oxysporum*
(B) *Ricinus communis* agaisnt *Macrophomina phaseolina*
(C) *Calotropis procera* against *Fusarium oxysporum*
(D) *Calotropis procera* agaisnt *Macrophomina phaseolina*

3.4 Dry Weight Analysis of Fungal Inhibition Under Enzyme–Extract Treatments

One-way ANOVA revealed highly significant effects of treatment level (T1–T3) on fungal dry weight of *Macrophomina phaseolina* and *Fusarium oxysporum* for both *Ricinus communis* and *Calotropis procera* extracts ($p < 0.001$). In *R. communis*, fungal biomass decreased significantly in a clear concentration-dependent manner, and Tukey's HSD confirmed that T1, T2, and T3 were all significantly different from each other and from the control ($p < 0.001$), following the inhibition order T3 > T2 > T1 > Control independent t-tests further showed that T3 differed very significantly from control ($p < 0.001$). *C. procera* also exhibited statistically significant, dose-dependent inhibition ($p < 0.001$), although the magnitude of biomass reduction was comparatively lower than *R. communis*. Bubble graph visualization of percentage inhibition demonstrated increasing bubble size and color intensity from T1 to T3, confirming maximum inhibition at T3 and supporting the quantitative findings. Notably, antifungal efficacy of both plant extracts was markedly enhanced when combined with the cell-free extract (CFE) of *Trichoderma harzianum*, with CFE + *R. communis* achieving 64.35% and 60% inhibition against *M. phaseolina* and *F. oxysporum*, respectively, and CFE + *C. procera* achieving 53% and 56% inhibition, respectively, indicating synergistic enhancement of antifungal activity through combined treatment (shown in Fig. 3 and Fig. 4).

Statistical Summary

Treatment	Mean ± SD
Control	33.1 ± 0.70
T1	24.8 ± 0.56
T2	18.6 ± 0.46
T3	11.8 ± 0.40

(A)

Treatment	Mean ± SD
Control	34.6 ± 0.72
T1	29.8 ± 0.76
T2	24.9 ± 0.62
T3	19.5 ± 0.60

(B)

Treatment	Mean ± SD
Control	37.57 ± 0.68
T1	30.40 ± 0.75
T2	22.60 ± 0.75
T3	14.67 ± 0.49

(C)

Treatment	Mean ± SD
Control	38.90 ± 0.70
T1	34.10 ± 0.75
T2	29.30 ± 0.75
T3	24.80 ± 0.75

(D)

Fig. 3. Statistical analysis of dry weight analysis.

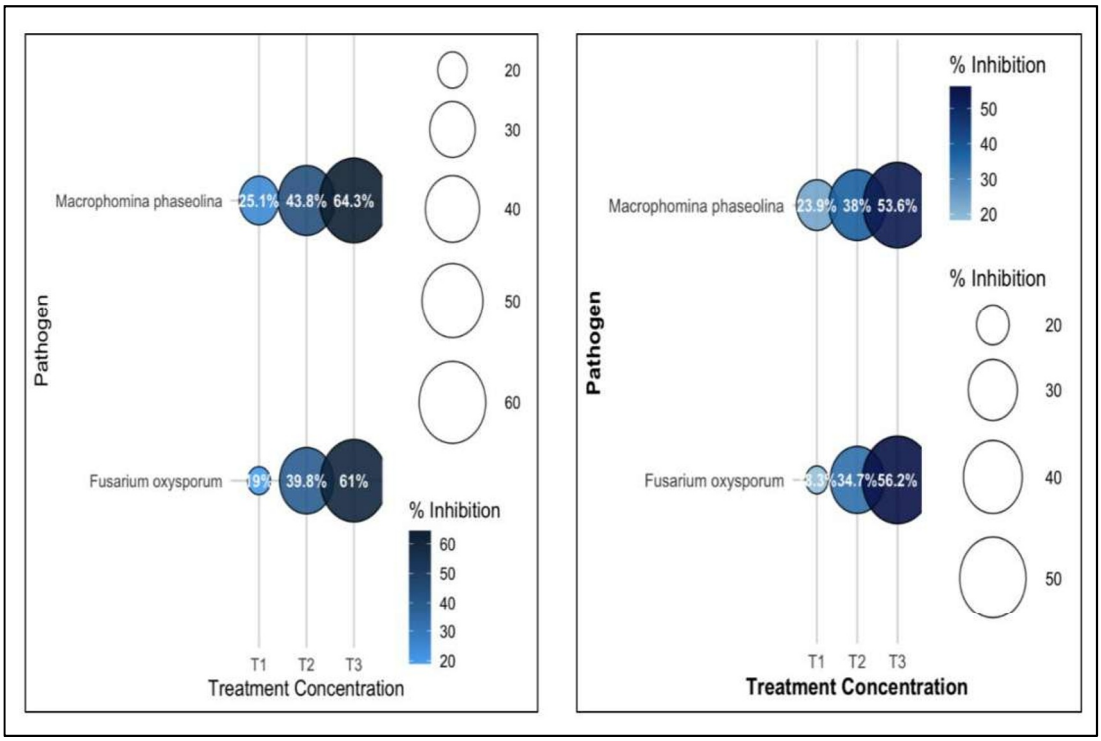

Fig. 4. Inhibition percentage of dry weight analysis.

3.5 Comparative Antifungal Efficacy of Individual and Combination Treatments at T3 Against *Macrophomina phaseolina* and *Fusarium oxysporum*

The data on the spider graph representation (Fig. 5) revealed a definite hierarchy of the antifungal efficacies in treatment of both *Macrophomina phaseolina* and *Fusarium oxysporum* at T3. The individual plant extracts, *Ricinus communis* showed the worst inhibitory effect, since it inhibited *F. oxysporum* 48% and *M. phaseolina* 44% and thus, *Ricinus communis* showed better intrinsic antifungal potential than *Calotropis procera* and *Azadirachta indica*. *A. indica* was moderately active in that it produced 40-30% inhibition towards *F. oxysporum* and *M. phaseolina*, respectively, and *C. procera* was less suppressive as it exhibited 33% and 35% inhibition towards *F. oxysporum* and *M. phaseolina*, respectively. It is interesting to note that the combination of plant extracts with the cell-free extract (CFE) of *Trichoderma harzianum* had an enormous effect, increasing the antifungal activity significantly. The significantly highest total inhibition was attained with the combination CFE + *R. communis* at 64% against *M. phaseolina* and 60% against F. oxysporum obviously exceeding the performance of each of the treatments. CFE + C. procera also showed a strong response, inhibiting 53% of *M. phaseolina*, and 56 % of F. *oxysporum*, which is a significant improvement over *C. procera*. These findings are confirmed by the spider graph visualization, in which the polygonal areas are enlarged to those with combination treatment, especially CFE + *R. communis*, which shows the highest levels of suppression. Taken together, all these findings indicate that combination treatment obtained notable improvement over individual extracts at T3, implying that the interaction between plant-derived bioactive compounds and extracellular metabolites found in the CFE of *T. harzianum* was synergistic. This type of synergy is probably based on complementary effects such as enzymatic degradation of fungal cell walls, membrane destabilization, and metabolic interference, which result in increased biomass reduction and growth inhibition. The strong activity of CFE + *R. communis* underlines its suitability as a biologically powerful and combined approach to the control of soil-borne pathogenic fungi.

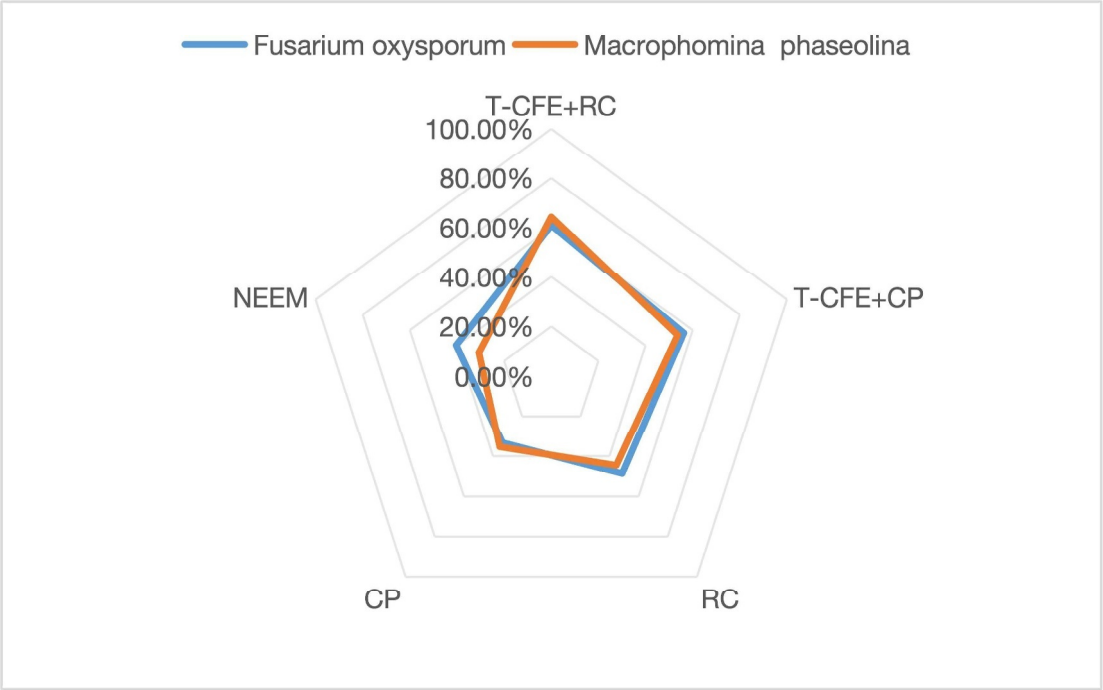

Fig. 5. Spider graph represents inhibition of all the combinations of extracts and CFE (T-CFE+ RC = Cell free extract of *Trichoderma harzianum + Ricinus communis,* T-CFE+ CP = Cell free extract of *Trichoderma harzianum + Calotropis procera,* RC = *Ricinus communis,* CP = *Calotropis procera*)

4. Discussion

The current study illustrates that there is a definite improvement in the disease management in phytomycobiology by combining plant bioactive compounds with the cell-free extract (CFE) of *Trichoderma harzianum* [22]. These findings were in agreement: Ricinus communis had significantly better antifungal activity than either *Calotropis procera, Azadirachta indica* and the antifungal activity of *Ricinus communis* was significantly improved in combination with CFE. This relative order of precedence is further supported by the spider graph visualization, which shows exactly that the CFE + *R. communis* treatment has occupied the greatest area of inhibition of both of the treatments under test, which makes it the most effective treatment of all the treatments under trial. The improved efficacy of the CFE-based combinations is a kind of product of PMB interaction and not a mere sum of the effects [23]. Compared to formulations that use live spores, CFE use eliminates variability due to fungal colonization without loss of biologically active metabolites and extracellular enzymes. CFE of *T. harzianum* has been reported to be composed of chitinases, β-glucanases, proteases, secondary metabolites, and other antifungal molecules that directly degrade the cell walls of fungi, interfere with the integrity of membranes, and suppress the metabolism of the pathogen (as shown in Fig. 6) [23,24]. The final formulation is a compound of the phytochemicals of *R. communis* (alkaloids, phenolics, flavonoids, tannins and saponins) which probably promotes multi-targeted stress through membrane destabilization, oxidative imbalance, enzymatic activity degradation and metabolic inactivation. Such a complementary biochemical attack can be attributed to the significant percentage of inhibition obtained at T3, in which CFE + *R. communis* activity was higher than that of only *R. communis*, and this finding demonstrates the better antifungal ability of CFE [24].

Notably, this research presents an efficient prospective PMB alternative to fungicides. The use of chemical fungicides is both effective, but is linked to environmental contamination, the development of resistance to pathogens, phytotoxicity as well as poor health effects on humans because of the presence of toxic residues. Plant extracts and microbial metabolites on the other hand are biodegradable, eco-friendly, and have multi-site modes of action, which minimises the chance of resistance emerging(as shown in Fig 6) [25]. The CFE-derived live spores are also free and add to the formulation stability and biosafety without any impact on antifungal effects. Other advantages of treatments based on CFE are that it induces plant systemic resistance, increases nutrient availability, improves rhizosphere microbial stability as well as lessening of chemical load in agroecosystems [27,28].
In general, the enhanced inhibition observed by CFE + *Ricinus communis* proves the prospect of such an integrated PMB solution as a more sustainable, biologically resilient, and less toxic substitute of the traditional fungicides. The analysis of comparative data shows a solid argument of using plant extracts in combination with bioactive metabolites of *Trichoderma harzianum* to improve antifungal activity using a synergistic effect, which provides the strategy as an integrated element of future integrated disease management programs [30].

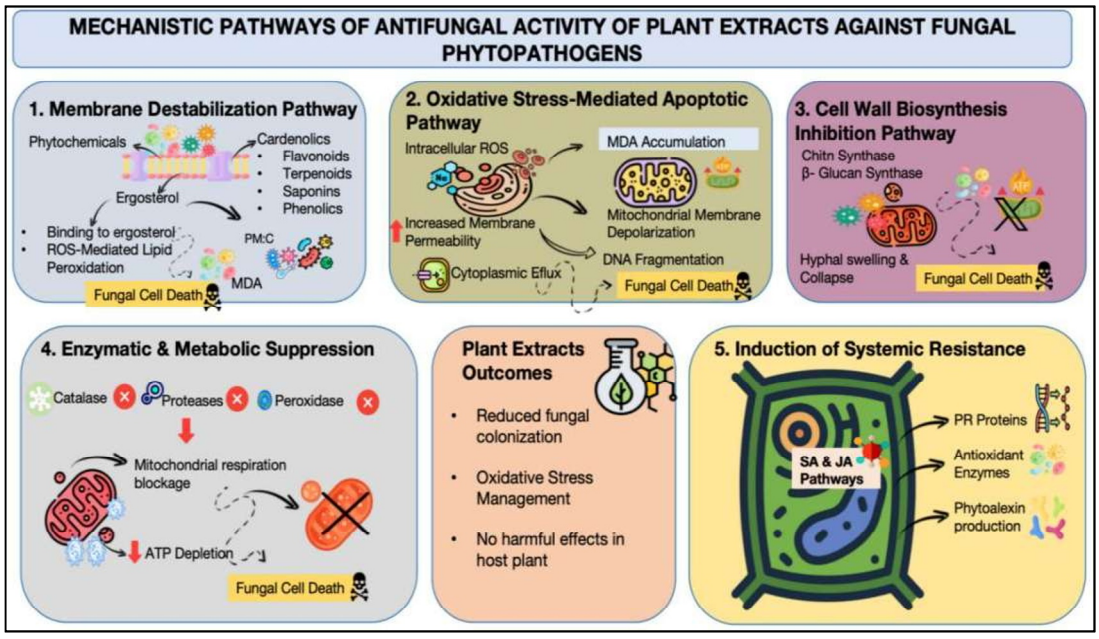

Fig. 6. Mechanistic pathways of antifungal activity of plant extracts against fungal phytopathogens. Source : [4,7,10,11,15, 20, 25].

5. Conclusion

The present study establishes that the addition of phytobiological extracts with the cell-free extract (CFE) of *T. harzianum* to antifungal activity boosts the antifungal activity against *Macrophomina phaseolina* and *Fusarium oxysporum* significantly. *Ricinus communis* was found to be the most intrinsically effective of the tested botanicals and its synergistic activity with CFE was the most effective especially at T3 point, thus suggesting a high synergy change between the two. The enhanced suppression is probably due to complementary processes of phytochemical caused membrane disruption and oxidative stress in addition to enzymatic degradation of cell walls of fungi by CFE-produced metabolites. In general, CFE + *Ricinus communis* formulation is one of the potential,

environmental-friendly alternatives to synthetic fungicides and contributes to the creation of the sustainable integrated disease management practices.

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Abbreviations
The following abbreviations are used in this manuscript:

CFE = Cell free extract
PMB = PhytoMycoBiological
PLE = Plant leaf extract

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