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MOLECULAR IDENTIFICATION AND FORMULATION OF MYCOHERBICIDES USING FUNGAL ISOLATES FOR EFFECTIVE CONTROL OF WATER HYACINTH

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Abstract– Water hyacinth (*Eichhornia crassipes*) is a pervasive aquatic weed that significantly impacts ecosystems and economies. This study aimed to identify fungal isolates with mycoherbicidal potential and develop effective formulations for controlling this invasive species. Genomic DNA from fungal isolates was extracted, and the 18srRNA region was amplified and sequenced. Phylogenetic analysis confirmed the identity of the isolates. The mycoherbicidal efficacy of these isolates was evaluated through in vivo and in vitro assays. Various liquid-based formulations were tested to optimize spore germination and seedling mortality, with Tween-80 emerging as the most effective adjuvant. Results demonstrated significant synergistic effects (up to 80%) when combining *Alternaria alternata* with other fungal pathogens, leading to enhanced disease progression on water hyacinth. The findings suggest that integrating multiple fungal species into a mycoherbicidal formulation could offer a promising strategy for managing water hyacinth infestations.

INTRODUCTION

Water hyacinth (*Eichhornia crassipes*), a native plant of the Amazon Basin, has become a global invasive species, causing severe ecological and economic issues (Villamagna and Murphy, 2010). Its rapid spread across water bodies leads to clogged waterways, loss of biodiversity, and disruptions in aquatic ecosystems (Patel, 2012). Traditional management methods, such as mechanical removal and chemical herbicides, have limitations including high costs and environmental impacts, prompting the exploration of biological control options (Charudattan, 2001). Fungal pathogens offer a promising alternative due to their specificity, environmental safety, and potential for sustainable application (Dagno *et al*., 2012). Previous studies have identified various fungal species with potential biocontrol properties, but more comprehensive studies are needed to identify and optimize effective fungal isolates and their formulations for field application (Ray and Hill, 2012). This study aims to fill these gaps by identifying fungal pathogens from water hyacinths, characterizing them at the molecular level, evaluating their pathogenicity, and developing effective formulations to enhance their biocontrol efficacy. Molecularly characterize fungal pathogens isolated from water hyacinth using ITS sequencing and phylogenetic analysis (Schoch *et al.,* 2012). Evaluate the pathogenicity of the identified fungal isolates against water hyacinth (Shabana *et al.,* 2003). Develop and test integrated and liquid-based formulations to optimize the efficacy of the most promising fungal pathogens as mycoherbicides (Ash, 2010). Specific fungal isolates from water hyacinth exhibit significant pathogenicity and, when optimally formulated, can effectively serve as biocontrol agents. This study contributes to sustainable invasive species management by providing a detailed characterization of fungal pathogens specific to water hyacinth and optimizing their application as biocontrol agents (Hoagland *et al*., 2007).

MATERIALS AND METHODOLOGY

Molecular identification of fungal isolates

Genomic DNA was isolated and its purity and concentration were assessed using a Denovix DS-11 spectrophotometer. The ITS region was amplified

with universal primers (ITS1 and ITS4) (White *et al*., 1990) using Taq DNA Polymerase 2xMaster Mix RED on a GeneAmp PCR System 9700. The resulting PCR amplicon underwent Exo-SAP purification (Bell, 2008), followed by bi-directional sequencing with a BDT V3.1 Cycle sequencing kit on an ABI 3730 Genetic Analyzer. Sequences were assembled into a consensus using Gene Tool software and analyzed for homology with the BLAST program (Altschul *et al*., 1990), identifying the top ten matches based on maximum identity and phylogenetic results.

Phylogenetic analysis

Nucleotide sequences showing homology from the BLAST search were designated as reference sequences and downloaded from GenBank. These sequences, along with those from the isolated fungi, were aligned using the Cluster X program (O'Leary *et al*., 2015). A phylogenetic tree was then constructed with MEGA 5.0 using the neighbor-joining method based on ITS gene sequences. The tree's reliability was tested with 1000 bootstrap analyses to ensure clade stability (Choi *et al*., 2019).

Formulation of mycoherbicide

The experiments aimed to evaluate various adjuvants and solid carriers to develop an effective mycoherbicide formulation. The study also investigated the synergistic potential of *Alternaria alternata* combined with other water hyacinth pathogens, using the dual culture method to assess compatibility (Boyette *et al*., 1979).

Liquid-based formulation

Five formulating agents-Tween 80, Tween 20, gelatin, soybean oil, Triton X-100, and sorbitol-were tested in a mycoherbicide formulation at 0.5% concentration. A seedling bioassay determined the herbicidal potential, with distilled water as the control. Treatments were performed in triplicate, and disease severity was rated on a scale from 0 (no disease) to 5 (81-100% plant damage). Spore germination was assessed after 7 days using a hemocytometer (Pandey and Kalam, 2016).

Table 1. Accession Numbers of Fungal Isolates from WH Samples

S.No.	Isolates	Strains	Accession no.
1.	WH-1	*Cladosporium tenuissimum*	MZ377141.1
2.	WH-2	*Fusarium equiseti*	MZ377143.1
3.	WH-3	*Curvularia lunata*	MZ377144.1
4.	WH-8	*Penicillium oxalicum*	MZ377238.1
5.	WH-11	*Nigrospora oryzae*	MZ377239.1
6.	WH-15	*Alternaria alternata*	PP994478.1

Integrated formulation

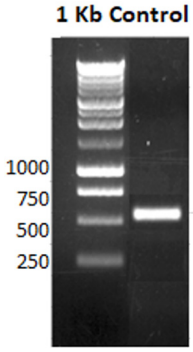
This study evaluated the synergistic interaction between *Alternaria alternata* and other water hyacinth pathogens to develop a more effective mycoherbicide. Compatibility was tested using the dual culture method. Pure fungal cultures were grown on Petri dishes with Richard medium, with one test pathogen and another pathogen placed 3.5 cm apart. This process was repeated with three fungal isolates to assess antagonistic effects. Control dishes contained only the test pathogen. Plates were incubated at 28 °C ± 1 °C for seven days (Shi *et al*., 2019).

RESULTS AND DISCUSSION

The current study focused on fungal isolates from water hyacinth plants showed a wide range of pathogenic fungi, *viz. Cladosporium tenuissimum, Fusarium equiseti, Curvularia lunata, Penicillium oxalicum, Nigrospora oryzae*, and *Alternaria alternata*. These identifications were done based on ITS region amplification and Sanger sequencing followed by subsequent phylogenetic analysis. BLAST for sequence homology and CLUSTAL W for phylogenetic tree analysis, further confirm those of Shabana and Mohamed (2005), and the application methods were deemed to be reliable for fungi identification. In the formulation of mycoherbicides, much attention was directed to sound formulation packages to ensure the activity of the microbial agent in the field. As noted by Soper and Ward (1981) and Greaves *et al.,* (1998), the development of microbial herbicides must be such that the formulation should provide enough moisture for fungal germination and subsequent growth. The findings underscore the challenges posed by environmental conditions and the need for formulations that can withstand these challenges to ensure consistent efficacy.

Molecular characterization of the isolated fungi from the water hyacinth plant

DNA was extracted from mycelium and amplified using ITS1 and ITS4 primers. The use of BLAST for sequence homology and the construction of phylogenetic trees using tools like CLUSTAL W is a



Lane1: 1KB DNA ladder
Lane 2: ITS amplicon (Control)

Fig. 1. Represents a single PCR product of fungal ITS region on 2% agarose gel

well-established approach to determining evolutionary relationships among fungal species.

Molecular Characterization of WH-1

The fungal sample isolate no WH-1, upon analysis, was identified as *Cladosporium tenuissimum*. This identification was determined through a combination of nucleotide homology and phylogenetic analysis. The process involved isolating genomic DNA from the sample, amplifying the ITS region using universal primers, and performing bi-directional sequencing. The resulting sequences were compared against the NCBI GenBank database, where *Cladosporium tenuissimum* emerged as the closest match based on significant alignments. This finding is further supported by the construction of a phylogenetic tree, which places the sample within the *Cladosporium* genus, confirming its identity as *Cladosporium tenuissimum*

The evolutionary history was inferred by using the Maximum Likelihood method and the Tamura 3-parameter model. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura 3 parameter model and then selecting the topology with superior log likelihood value. This analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There was a total of 712 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

Molecular Characterization of WH-2

Upon analysis, the fungal sample isolate no WH-2 revealed that the organism shows significant similarity to *Fusarium equiseti*. This conclusion is based on nucleotide homology and phylogenetic analysis conducted on the fungal DNA extracted from the sample. The DNA was amplified using universal ITS primers, and the sequences obtained were compared against the NCBI GenBank database. The resulting high similarity scores and the constructed phylogenetic tree support the identification of the organism as *Fusarium equiseti*, which is known for its pathogenic potential in plants and various environmental settings.

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Tamura 3-parameter method

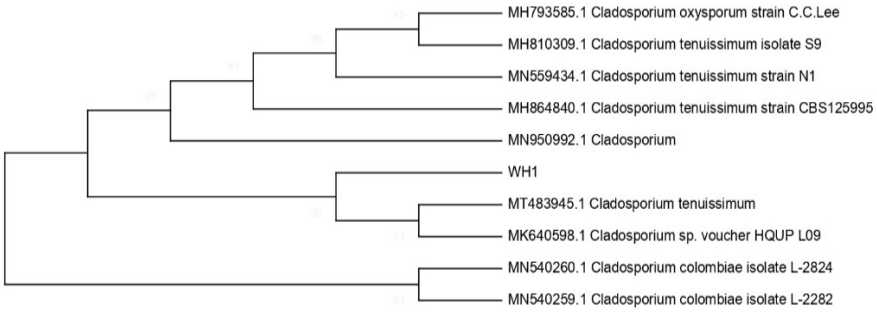


Fig. 2. Phylogenetic relationship of isolate WH-1 showing homology with the analyzed sequences by maximum likelihood method based on 18S rRNA gene sequence.

and are in the units of the number of base substitutions per site. This analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 567 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

Molecular Characterization of WH-3

The fungal sample isolate no WH-3, upon analysis, revealed that the organism is closely related to *Curvularia lunata*. This identification was determined through a comprehensive analysis that included DNA extraction, amplification of the ITS region, and subsequent sequencing. The sequences were compared against the NCBI GenBank database, and the results showed a significant match with *Curvularia lunata*, a fungus known for its pathogenic effects on various plants. The identification was further confirmed through phylogenetic analysis, which placed the sample in close relation to other known strains of *Curvularia lunata*, thereby providing strong evidence of its identity.

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Tamura 3-parameter method and are in the units of the number of base substitutions per site. This analysis involved 9 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1061 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

Molecular Characterization of WH-8

The fungal sample isolate no WH-8, upon analysis, revealed that the organism has identified the organism as *Penicillium oxalicum*. This conclusion was reached through a detailed process involving the isolation of genomic DNA, amplification of the ITS region, and subsequent sequencing. The sequences were compared against the NCBI GenBank database using BLAST analysis, which revealed a significant similarity to *Penicillium*

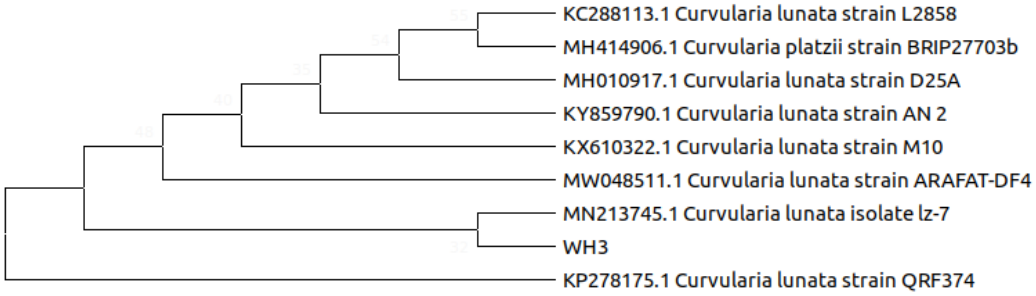


Fig. 4. Phylogenetic relationship of isolate WH-3 showing homology with the analyzed sequences by maximum likelihood method based on 18S rRNA gene sequence.

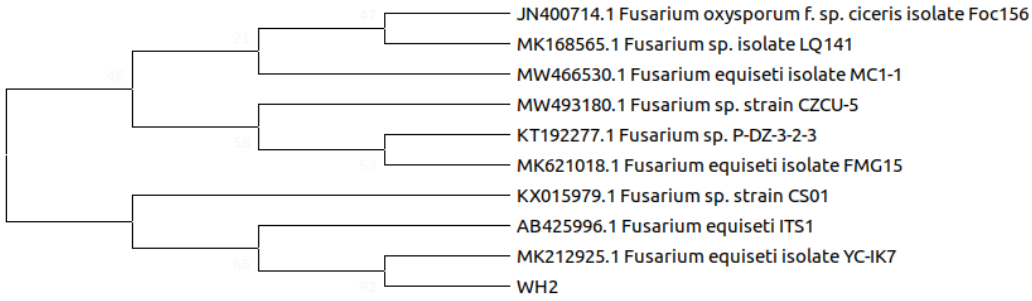


Fig. 3. Phylogenetic relationship of isolate WH-2 showing homology with the analyzed sequences by maximum likelihood method based on 18S rRNA gene sequence.

oxalicum. This identification was further supported by phylogenetic analysis, which confirmed the close evolutionary relationship of the sample to other known strains of *Penicillium oxalicum*.

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Tamura 3-parameter method and are in the units of the number of base substitutions per site. This analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1081 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

Molecular Characterization of WH-11

The fungal sample isolate no WH-11, upon analysis, revealed that the organism has identified the organism as *Nigrospora oryzae*. This conclusion was derived through a detailed nucleotide homology and phylogenetic analysis. The ITS region of the genomic DNA was amplified and sequenced, with the resulting sequences being compared against the NCBI GenBank database using BLAST analysis. The analysis revealed a significant similarity with *Nigrospora oryzae*, a known phytopathogenic fungus. The identification was further confirmed by phylogenetic tree analysis, which showed the sample clustering closely with other *Nigrospora oryzae* strains, reinforcing the accuracy of the identification.

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were computed using the Tamura 3-parameter method and are in the units of the number of base

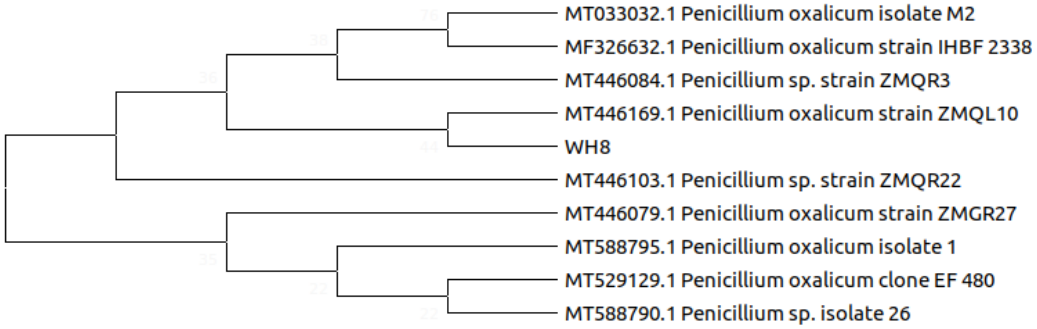


Fig. 5. Phylogenetic relationship of isolate WH-8 showing homology with the analyzed sequences by maximum likelihood method based on 18S rRNA gene sequence.

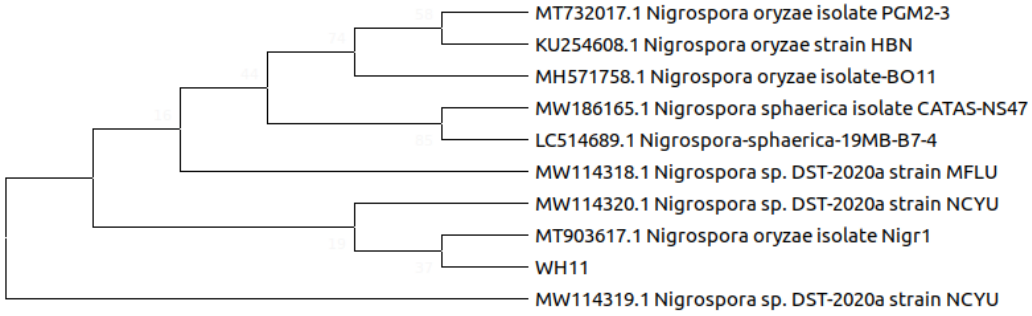


Fig. 6. Phylogenetic relationship of isolate WH-11 showing homology with the analyzed sequences by maximum likelihood method based on 18S rRNA gene sequence.

substitutions per site. This analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 776 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

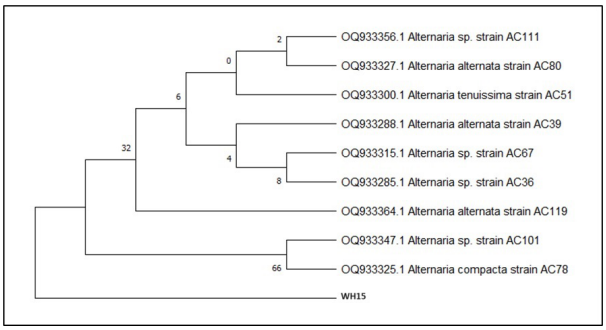


Fig. 7. Phylogenetic relationship of isolate WH-15 showing homology with the analyzed sequences by maximum likelihood method based on 18S rRNA gene sequence.

Molecular Characterization of WH-15

The fungal sample isolate no WH-15, upon analysis, revealed that the organism has identified the organism as *Alternaria alternata* based on ITS sequence analysis. The analysis involved isolating DNA from the provided culture, amplifying the ITS region using specific primers, and performing forward and reverse sequencing. The resulting consensus sequence was compared against sequences in the NCBI GenBank database using BLAST, where *Alternaria alternata* emerged as the closest match with a high degree of similarity. This identification was further supported by a phylogenetic analysis, which placed the sample within the *Alternaria* genus, specifically aligning it with strains of *Alternaria alternata*.

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 50% of bootstrap replicates are collapsed. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. This analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1120 positions in the final dataset. Evolutionary analyses were conducted in MEGA11.

Formulation of mycoherbicide

The general considerations and criteria of fungal formulation given by Soper and Ward (1981) are still the primary source of guidance. According to them

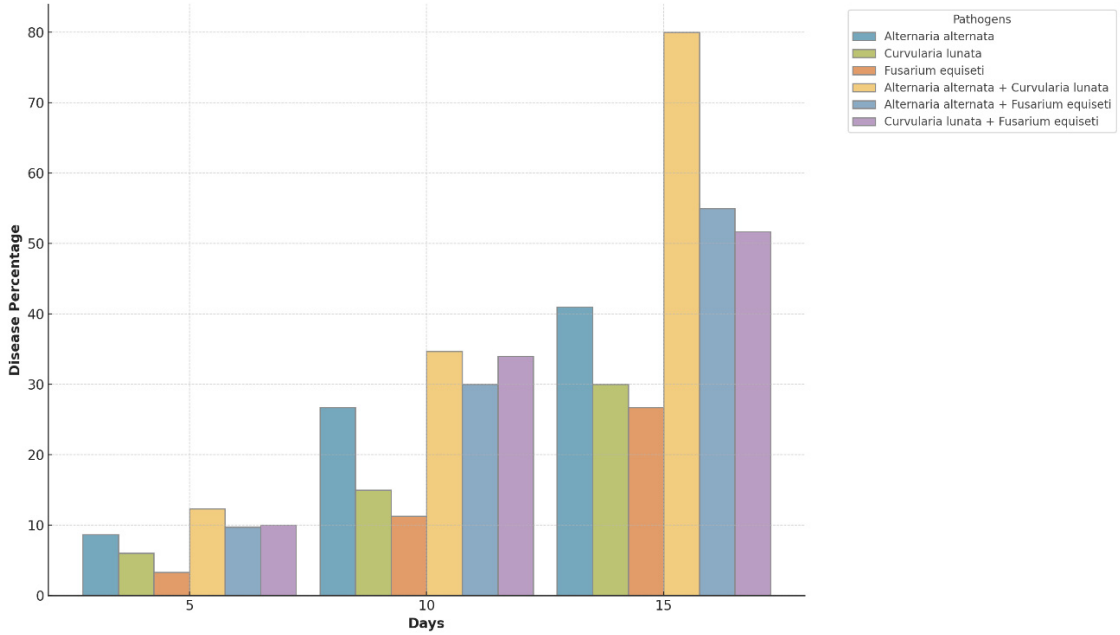


Fig. 8. Bar chart showing disease percentage over time (5, 10, 15 days) in water hyacinth. Disease severity peaks at 15 days, especially with multiple pathogens, indicating a synergistic effect.

formulation attempting to provide the correct combination of the active and final components is crucial for agricultural application. Microbial herbicide efficacy in the field is constrained, particularly, by the unsuitable climatic conditions that often exist when the product is applied. In particular, the agent depends on having adequate moisture present for sufficient time to promote and sustain its germination and growth on the target plant it has penetrated and infected the tissues. This moisture may be provided in the form of dew or rain, neither of which is sufficiently reliable to guarantee efficacy or by formulation. Such formulation must be robust and designed to meet stringent criteria that ensure enhancement of microbial health and performance relative to the unformulated agent (Greaves *et al*., 1998) acknowledged that other factors, notably formulation; contribute significantly to the failure to translate laboratory success into practical success in the field.

Integrated formulation

In natural plant ecosystems, pathogens do not exist in an axenic state but rather exist with a diverse epiphytic microflora. Whilst one organism alone may initiate infection, the speed and severity of disease progression may be influenced by the biota surrounding the infection court (Cother, 1992). In the majority of diseases, single-entity pathogens are the norm. However, adding another pathogen to the biological spray mixtures considerably increases the potential for manipulating phylloplane ecosystems and subsequent disease initiation. Templeton and Heiny (1989) suggested that several isolates of one pathogen or several species of pathogens each having slightly different environmental requirements could be mixed in the formulation to ensure that at least one would encounter the optimal environmental window. Hasan and Ayers (1990) reported that interaction between the biotroph and necrotrophs occurs at the infection site of biotrophs, where infection by one pathogen makes the host

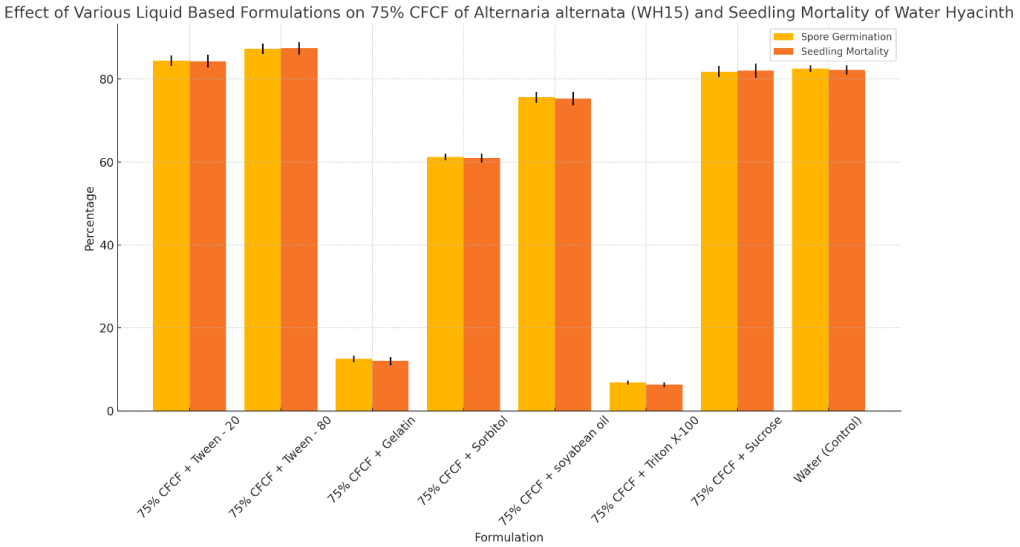


Fig. 9. Graphical representation of the effect of various liquid-based formulations on 75% CFCF of *Alternaria alternata* (WH-15) and seedling mortality of water hyacinth at 28 ± 1°C

Table 2. Effect of pathogens and their combination on disease percentage in water hyacinth

S. No.	Pathogen	Disease percentage		
		5th day	10th day	15th day
1.	*Alternaria alternata*	8.7	26.7	41.0
2.	*Curvularia lunata*	6.0	15.0	30.0
3.	*Fusarium equiseti*	3.3	11.3	26.7
4.	*Alternaria alternata* + *Curvularia lunata*	12.3	34.7	80.0
5.	*Alternaria alternata* + *Fusarium equiseti*	9.7	30.0	55.0
6.	*Curvularia lunata* + *Fusarium equiseti*	10.0	34.0	51.7

Table 3. Effect of various liquid-based formulations on 75% CFCF of *Alternaria alternata* (WH-15) and seedling mortality of water hyacinth at 28 ± 1 °C

S. No.	Formation	Spore germination (%)	Seedling Mortality (%)
1.	75% CFCF + Tween - 20	84.4 ± 1.3	84.3 ± 1.5
2.	75% CFCF + Tween - 80	87.3 ± 1.3	87.4 ± 1.5
3.	75% CFCF + Gelatin	12.5 ± 0.8	12.0 ± 1.0
4.	75% CFCF + Sorbitol	61.2 ± 0.8	61.0 ± 1.1
5.	75% CFCF + soyabean oil	75.6 ± 1.3	75.3 ± 1.6
6.	75% CFCF + Triton X-100	06.8 ± 0.5	06.3 ± 0.6
7.	75% CFCF + Sucrose	81. 8 ± 1.4	82.0 ± 1.7
8.	Water (Control)	82.5 ± 0.8	82.2 ± 1.1

Values are presented as mean ± SD of three observations
Germination was observed after 7 days of incubation.

more susceptible to secondary infection. Such type of synergistic relationship of two pathogens provides biological and economic feasibility of the use of the mixtures of two or more fungi for effective control of one or more weeds could go a long way to ensure the success of any biocontrol program. Therefore, an experiment was carried out to investigate the compatibility of *Alternaria alternata* with other potential fungal pathogens of water hyacinth under laboratory conditions.

In vivo, the disease percentages were greater on water hyacinth leaves when a combination of pathogens was used than the individual pathogens. The *Alternaria alternata* + *Curvularia lunata* combination resulted in maximum disease development followed by *Alternaria alternata, Curvularia lunata*, *Alternaria alternata* + *Fusarium equiseti* and *Curvularia lunata* + *Fusarium equiseti* combinations gave similar results. Similar observations have been made by several other workers (Van Den Brink *et al.,* 1998; Pandey, 1998). Thus, it can be concluded that better control of the weed can be achieved using combinations of pathogens.

Liquid-based formulation

Liquid-based formulations evaluated during the present studies showed the significant pathogenic effect of *Alternaria alternata* (WH-15), a mycoherbicidal agent for water hyacinth. It was observed that all the adjuvants promoted spore germination to a great extent except Triton X-100 and Gelatin (Table 3). Germination was highest in the presence of Tween–80 (87.3%) which was followed by Tween–20 (84.4%). Vegetable oil and sucrose also supported the spore germination.

Significant differences in the virulence (seedling mortality) of *Alternaria alternata* (WH-15)were also recorded. Maximum seedling mortality was observed at 7th (days after treatment) when Tween-80 was used as an adjuvant. It was followed by Tween-20, sucrose, water alone, and soyabean oil. Triton X-100 and gelatin formulations gave negligible seedling mortality. More or less similar trends have also been recorded by earlier workers (Greaves *et al*., 1998).

CONCLUSION

The molecular identification and phylogenetic analysis of fungal isolates revealed several species with significant mycoherbicidal potential, including *Cladosporium tenuissimum*, *Fusarium equiseti*, *Curvularia lunata*, and *Alternaria alternata*. The formulation studies demonstrated that the choice of adjuvant plays a critical role in spore germination and seedling mortality, with Tween-80 emerging as the most effective. Moreover, the combination of *Alternaria alternata* with other fungal pathogens resulted in enhanced disease severity on water hyacinth, indicating a synergistic interaction that could be exploited for more effective weed control. These findings underscore the potential of developing integrated mycoherbicidal formulations as a viable strategy for managing water hyacinth infestations, offering a sustainable alternative to conventional methods. Further field trials are recommended to validate these formulations under natural conditions.

Conflict of Interest- None

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