CHAPTER 3:

EVALUATION OF THE BIOCONTROL POTENTIAL OF FUNGAL PATHOGEN AGAINST THE WEED

3. EVALUATION OF THE BIOCONTROL POTENTIAL OF FUNGAL PATHOGEN AGAINST THE WEED

3.1 Introduction

Several control mechanisms in the form of manual, mechanical, chemical and biological methods have been executed to check the high rates of infestation of waterhyacinth, with varying results (Charudattan et al. 1996). However, various environmental and economic challenges are associated with these latter mentioned methods. Over the last few decades, lot of emphasis has been on adapting eco-friendly measures, where several biocontrol agents including arthropods and plant pathogens have been released around the world to control the weed (Ray and Hill 2013a, Firehun et al. 2015). But in its range of introduction, the absence of control agents has led to further uncontrolled flourishment of waterhyacinth. The biological control agents have proved their excellence in the control of waterhyacinth in many locations around the world including India (Center 1994 Jayanth 1988 Coetzee et al. 2011, Dutta and Ray 2017). Though biological control of weeds using arthropods and pathogens single-handedly has gained considerable importance over the last five decades due to their eco-friendly, host-specific and effective means of weed control, these agents have often been found to be slow and unpredictable (Ray and Hill, 2016). Hence, more efforts are required to improve their biocontrol efficacy.

Two waterhyacinth weevils - *Neochetina bruchi* (Hustache) and *N. eichhorniae* (Warner) (Coleoptera: Curculionidae) have been an important and impactful biological control agents against waterhyacinth (Center et al. 1999, Julien et al. 1999). The weevils have similar kinds of performance, where they in their adult stage, cause damage to the host by feeding on the epidermal tissues of the laminae and petiole and scrapping off the cuticle and part of the mesophyll tissue (Ray et al. 2009). The larval stages of the weevils, in return, are endophagous in nature. They tunnel bored inside the petioles while feeding and progress towards the base and into the crown where they then, often feed on developing auxiliary buds before they go in for their pupation phase of development in the roots (Julien 2001). Thus, feeding by *Neochetina* spp. induces shoot mortality by constricting the plant height, biomass, and also dampens reproduction (Center et al. 1999). Uncountable feeding scars breaks the plant integrity by removing massive masses of epidermal tissue, which leads to increasing water loss,

and making the plant vulnerable to more pathogen attack (Julien 2001). Apart from the weevils, another arthropod biocontrol agents, a mite - *Orthogalumna terebrantis* Wallwork (Acarina: Galumnidae) has also shown promising results (Jayanth and Visalakshy 1989, Julien 2001, Marlin et al. 2013), on being used against waterhyacinth. The host-specific mites affect the growth and reproductive rate of waterhyacinth, eventually weakening it that leads to its death. The nymphs of this mite, clubbed together to form passageways between the parallel veins of the laminae from which emerge out as adults. Colonies of mite cause leaf discoloration in the form of chlorosis and desiccation, finally leading to the death of waterhyacinth (Julien 2001).

Apart from arthropods, studies show that pathogens can cause significant reduction in waterhyacinth biomass, especially following insect attack or natural disease outbreak (Ray and Hill, 2012b). Several studies on isolation, identification and biocontrol potential of the fungi have been tested in various infested areas worldwide (Daddy et al. 2003, Freeman et al. 1981, Naseema and Balakrishnan 2001, Okunowo et al. 2008, Praveena and Naseema 2006, Ray 2008). However, the continual survey and lookout of several new species can reduce the stress on limited species and broaden the list for agents against this weed, apart from the some of the most commonly used biocontrol agents like various species of *Alternaria, Acremonium,* and *Fusarium* etc. Pathogens, which are also native or endemic, are comparatively found to be more potent and ideal for the development of biocontrol agents (Cuda et al. 2008). Earlier several phytopathogenic fungi were isolated and identified from diseased lesions and/or patches on various parts of waterhyacinth growing in the vicinity of Kolkata, India. Proper identification of these phytopathogens were performed using both morphological and molecular works (as stated previously in the earlier **Chapter 2**). The isolated fungi would be tested for their pathogenecity potency against the weed, in this Chapter to under the efficacies in their disease causing potential and severity.

Studies involving interaction with other biocontrol agents apart from phytopathogens gives a glimpse into a reality. Various previous literature studies have shown cumulative or synergistic effects of the feeding of *Neochetina* along with the pathogenicity of fungi against waterhyacinth (Moran 2004, Martínez and Gómez Balandra 2007, Ray and Hill 2012b). But a review by McEvoy and Coombs (2000),

contrarily reports that the cumulative impact of agents might not always be favourable. Several possible negative effects may result due to multitude of factors with the release of numerous agents, altering the biological control programme from beneficial to less effectivity and risky. Therefore, it is difficult to predict the effect of such interactions on the host plants. Pathogenicity caused agents may orient the biochemistry of the host plant that can in return alter preferential consumption of infected tissue by the host herbivores (Ramsell and Paul 1990, Dutta and Ray 2017) with reduced palatability of plant tissue (Karban et al. 1987), by inducing the accumulation of high concentrations of deterring compounds (Piening 1972). Therefore, studies on inter-specific interactions are essential before releasing them in the field, in those combinations, for better biocontrol of the host, waterhyacinth.

Not just interaction among the agents themselves, but also with the surrounding nature was important, prior to the release of agents, because detrimental effects on surrounding species, can leave an unbalancing effect on the nature, because the risks involved in initiation can never be ruled out (Dutta et al. 2015). Host-range testing schemes were developed to test the specificity and spectrum of the phytopathogen's effect and used for assessing the safety of non-target plant species of similar ecological niches and economic status (Wapshere 1974). Therefore, the host-range test was carried on 67 plant species, belonging to 30 families, prior to releasing them in the field condition.

The phytopathogens thus when tested and found fit for the application, amongst the surrounding important flora and other biocontrol agents, are best for application to control the target weed. Hence, an initiative has been undertaken during the present times to enhance the biodiversity of phytopathogens from waterhyacinth in India, to control the host, with better efficacy in biocontrol potentiality and host-specificity.

3.2. Materials and methods

3.2.1 *Pathogenicity test of the fungi on the host-weed, waterhyacinth*

3.2.1.1. **Test plant preparation**

Plants, used for experimentation were collected, from various waterhyacinth-infested water bodies, at their early growing stages and grown in big plastic tanks as stock culture at Presidency University, Kolkata, India. The tanks were loaded with tap water supplemented with farmyard manure and a slow fertilizer of N:P:K in 15-3-12 proportion. A commercial (13 % Fe) iron chelate was also added to the water, for maintaining the iron balance for plant uptake, at 2 gm for every 23 l of water. An insecticide, Malathion (Kombat [Pty] Ltd) were sprayed, to keep them free from insect infestation, as per requirement. Plants were kept in the growth chamber, where temperature of 26 ^{o}C and relative humidity (RH) of 75 to 85 %, in plastic pots (18 mm dia) filled with fertilized water, were maintained prior to using them for experiments.

3.2.1.2 **Fungal mycelial and spore suspension preparation**

Cultures of different fungal strains, isolated from infected leaves of waterhyacinth found during periodical surveys from various water bodies in and around Kolkata, were collected. For all the experiments, 14 days old culture of the test fungi, grown in 90 mm potato dextrose agar (PDA) in Biological Oxygen Demand (BOD) incubator at 27 ^{o}C at a photoperiod of 12 hours, were used. The Petri plates were flooded with sterile distilled water and the fungal spores and mycelial mat were scraped out. A sterile spatula was used to obtain a fungal suspension containing 10 g of scraped material (spores and mycelia) to which 50 ml of sterile distilled water was added.

The fungal conidial suspension was visualized to contain about 10^5 - 10^7 spores/ ml using the haemocytometer counting technique (Singh and Singh 2007). Tween 20 (Oxysorbic polyxyethylene sorbitan monoleate), used as a surfactant, was added at the rate of 0.05 ml/ 50 ml, to the collected conidial suspension (Ray 2008).

Freshly prepared spore suspension of the fungi was then applied on the plants, previously grown in plastic tubs.

3.2.1.3. **Application of spore suspension**

To understand the disease-causing potential of the fungi, the isolates were tested for their infection-causing ability against waterhyacinth plants in vitro, initially by detached leaf bioassay followed by whole plant bioassay, under laboratory and greenhouse conditions, using fungal mycelium, spores and metabolised broth.

3.2.1.3.1. Detached leaf assay

Leaves for detached leaf assay were collected from plants of between 6 and 10 weeks old. The leaves were cut with a portion of petiole left to be covered with moist cotton and silver foil (**Figure 3.1**), to prevent the leaves from withering during the time of the experiment. The set-up was placed on Petri plates, lined with moist filter paper, to maintain humidity. In the first trial, the fungus spore suspension was applied on the piece of waterhyacinth leaf and left on moist filter paper in a Petri plate, to maintain the humidity.

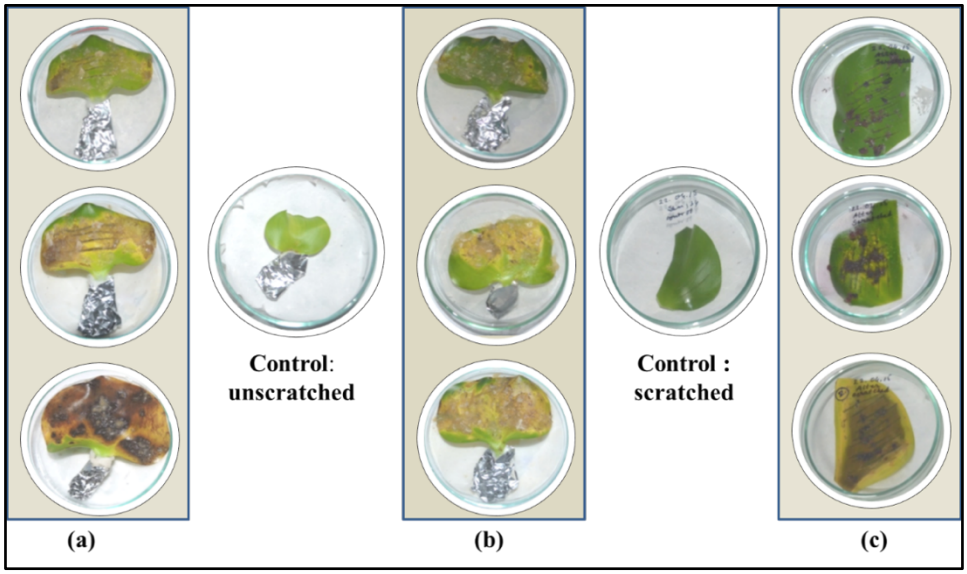


Figure 3.1: Example of Detached leaf assay on waterhyacinth leaves infected by (a) *Fusarium oxysporum* (b) *Fusarium roseum* and (c) *Alternaria alternata* at 24, 72 and 120 hours of incubation.

3.2.1.3.2. Whole plant bioassay

For the second round of trial, young water hyacinth plants acclimatized at the greenhouse garden of Presidency University, Kolkata as mentioned earlier, were taken for the experiment. Healthy individual plants, collected from these tanks, were washed

vigorously under the running tap water following with sterile distilled water wash, before being used for the pathogenicity trials. They were wiped with a tissue, dipped in 70 % alcohol, and shifted to tap water filled small tubs, pre-supplemented with fertilizer and added as and when required (**Figure 3.2**).

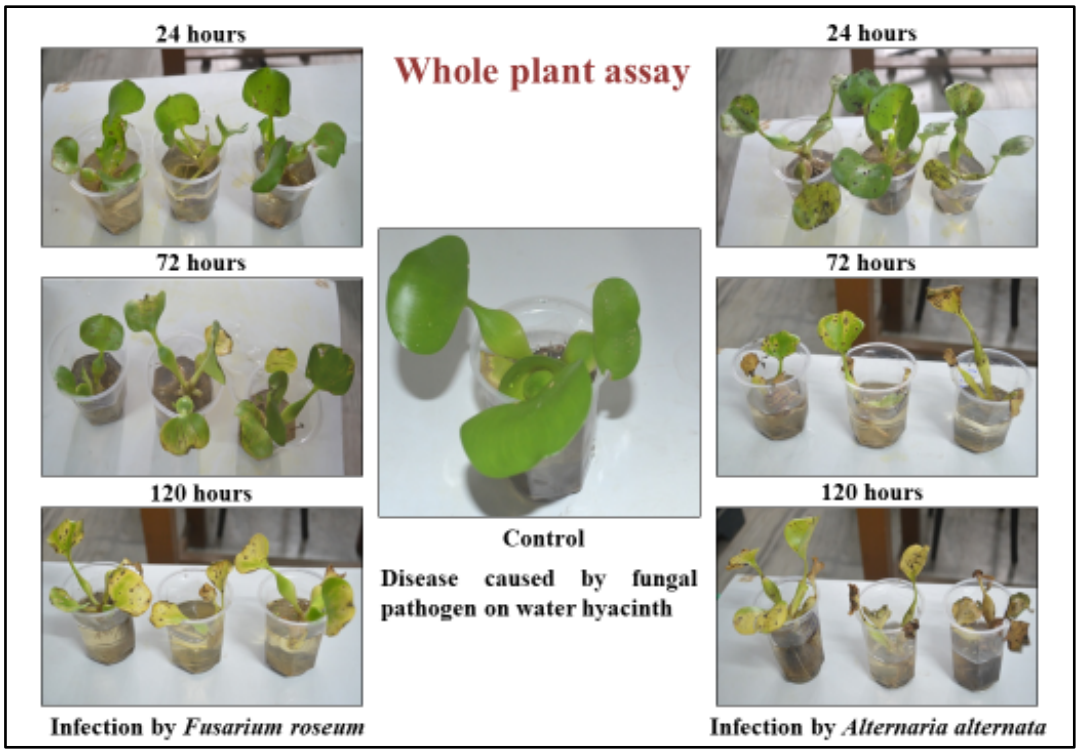


Figure 3.2: Example of whole plant bioassay on waterhyacinth leaves infected by (a) *Fusarium roseum* and (b) *Alternaria alternata* at 24, 72 and 120 hours of incubation.

After inoculation, the plants were covered with clear polyethylene bags (**Figure 3.3**), for the maintenance of relative humidity of around 70-80 %.

Figure 3.3: The plants after inoculation, were covered with clear polythene bags, to maintain the desired humidity

The set-up was kept in growth racks with a photoperiod of 12 hours (**Figure 3.4**). Data was recorded, in the form of percentage, at an interval of 24 hours, where the disease's presence was determined based on the exhibition of disease symptoms (in form of necrosis or chlorosis or both) or damage of the number of leaves on the plant and all this data was based on visual observations. Records were taken and disease intensity was categorized as excellent (+++), good (++), poor (+), and no infection (–) based on the virtue of a pictorial disease scale (**Figure 3.5**) (Freeman and Charudattan 1984, Dutta and Ray 2017). The disease was scaled from 0 to 9 rating system where 0 = healthy and 9 = 90 % diseased area covered by necrotic spots or chlorosis on leaves, until the death or damage of almost all the plants.

Figure 3.4: Experimental set-up in a growth-rack, maintaining a photoperiod of 12 hours

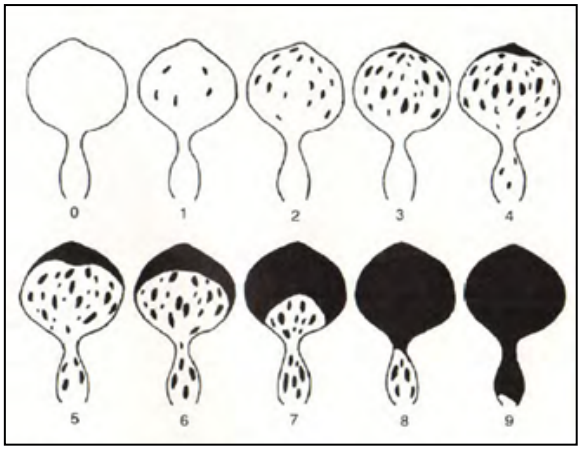


Figure 3.5: The pictorial disease scale used as reference for disease percentage calculation (Freeman and Charudattan, 1984)

Under controlled conditions, the ones displaying excellent disease-causing potential against waterhyacinth were selected for the next phases of the study.

Statistical analysis

The percentage data was recorded for evaluating the damage caused by different biocontrol agents, and were presented in terms of arithmetic mean and SEM (Standard Error of the mean). One-way analysis of variance (ANOVA), using statistical programme Statistica, was used to compare the disease intensity mean values of different isolates tested at each hour of exposure. A repeated measure of ANOVA was used to compare the means of disease density values at 24, 48, 72, 96, and 120 hours of exposure. The standard means of replicates of the treatments were matched with least significant difference (LSD) at 5 % level of significance (Sokal and Rohlf 1981).

3.2.2. *Host-range specificity test*

To understand the impact of phytopathogens on the surrounding ecological niche and other important flora, ecologically and economically important plants, related to waterhyacinth, were used to check the specificity of the various fungal pathogens on them, in order to understand the efficacy of these pathogens, prior to their application for the biocontrol of the host weed on its various infested sites, The potential fungal biocontrol agents of waterhyacinth (derived from the previous set of experiment results) were subjected to host range studies. Experimental inoculations of 67 plant species belonging to 30 families with mycelial suspension and crude metabolite of the respective fungi was done. For the host-range (**Figure 3.6**), study inoculum was

harvested by flooding these colonies with sterile water, disrupting the colony surfaces by scraping with the edge of a flamed microscope slide and filtering the resulting suspension through two layers of cheesecloth. Mycelial suspension was prepared using 10 mg of mycelial mat in 50 ml sterile distilled water while fungal crude metabolite was prepared using 21-day-old metabolized broth filtered from actively growing culture of the fungus. Conidial density of the mycelial suspension was measured using haemocytometer and it was adjusted to the required density by dilution with sterile water. For each plant species tested, two sets of test plants were prepared. Each set contained five pots and each pot contained between two and five plants. Test plants were propagated either from seed, cuttings or obtained from nursery. The test plants were inoculated when they had 3–5 fully mature leaves.

Damage or disease intensity was categorized as priory mentioned, based on virtue of a pictorial disease scale (Freeman and Charudattan 1984, Dutta and Ray 2017).

Figure 3.6: Set-up for host-range pathogenicity trials on several ecologically and economically important plants

3.2.3. *Arthropod- plant-pathogen interaction studies*

The study was done in order to understand the mutual impact of different biocontrol agents of waterhyacinth, among themselves, as well as on the biocontrol of the target weed. Fungal strains, having severe disease-causing potential were selected for this study and applied on waterhyacinth, singly and in different combinations with the arthropod biocontrol agents of the weed, including the chevroned waterhyacinth weevil or *Neochetina bruchi* Hustache (Curculionidae : Coleoptera) (NB), and waterhyacinth mite or *Orthogalumna terebrantis* Wallwork (Galumnidae : Acarina) (OT).

3.2.3.1. **Maintenance of arthropods**

Stock culture of OT and NB were maintained after collecting the arthropod infested waterhyacinth naturally growing in the weed infested water bodies. They were brought back and reared in several 50 l tanks in the institution's greenhouse with proper maintenance of conditions. Weevils rearing tanks were covered with fine mesh nets to prevent their escape. They were replenished with more waterhyacinth time to time, to feed and breed upon. To prevent mite invasion onto weevil attacked plants, the second set of tanks, where the former was maintained, was placed at a distance. All the tanks were reloaded with water and other fertilizers, as per requirement. For proper maintenance of healthy cultures and space, all dead leaves and daughter plants were removed from time to time. Adult arthropods were used from these maintained stock cultures for experimentation. Minute size of OT hindered individually transference, hence prior to experiment, leaf bearing them was placed in top of fresh leaves in the pots, for their total transfer from the detached leaf onto the plant used for the experiment. After the mites transferred onto fresh leaves, the old leaf was then removed.

Freshly prepared spore suspension of the potential fungi was sprayed on plants, until runoff. The fungal suspension was applied on waterhyacinth in various ways, either singly or in combination with NB (2 pairs/ plant) or OT (30–50/ plant) (**Figure 3.7**), obtained from their respective tanks, to observe the possible cumulative effect on the weed biocontrol as a whole.

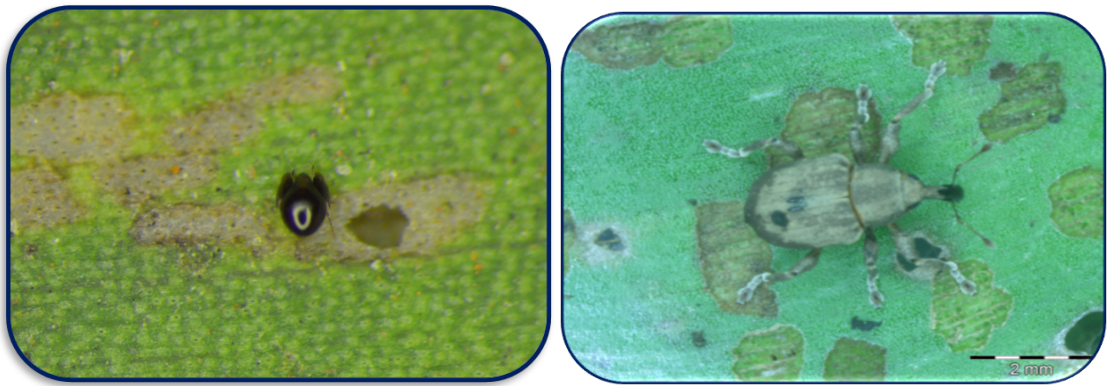


Figure 3.7: a. Waterhyacinth mite or *Orthogalumna terebrantis* Wallwork (Galumnidae : Acarina) (OT); **b**. Chevroned waterhyacinth weevil or *Neochetina bruchi* Hustache (Curculionidae : Coleoptera) (NB)

Experimental control included fresh unaffected plants, with no biocontrol agents. The four selected fungi (based on outcome of pathogenicity trials) and two arthropods were applied on waterhyacinth plants singly and in various combinations (OT, NB, FO, FO + OT, FO + NB, FR, FR + OT, FR + NB, PS, PS + OT, PS + NB, AA, AA + OT, AA + NB). Each set of treatments were replicated six times. [Based on the experiment result of the previous pathogenicity and biocontrol potential: AA- *Alternaria alternata*, FO- *Fusarium oxysporum* sp. *lycopersici* 4287, FR- *Fusarium verticillioides*, PS- *Paradendryphiella salina*]

Damage or disease intensity was categorized as excellent (+++), good (++), poor (+), and no infection (–) based on virtue of a pictorial disease scale (Freeman and Charudattan 1984, Dutta and Ray 2017).

3.2.3.2. **Host choice test**

Another set of experiment was conducted where a dozen weevils were placed in the middle of a test chamber, with six PS infected plants on one side and six fresh plants on the other. The entire set-up was then maintained at around 75 % relative humidity (RH) and a photoperiod of 12 hours, in plant growth chamber condition. NB were given free-choice and allowed to select their food plant, and not placed on any of the test plants. After a certain period of time, the feeding scars were counted (Ray et al. 2008b) from the two sets of plants used.

Statistical analysis

The study was replicated six times and analysis of variance (ANOVA) was performed using statistical programme Statistica. The experimental means were compared with least significant difference (LSD) at 5 % level of significance.

3.3. Results

3.3.1 *Pathogenicity test of the fungi on the host-weed, waterhyacinth*

Among the 152 isolates, 48 isolates, among which many reported for the first time, (**Table 3.1**) that caused the effective disease to waterhyacinth leaf pieces in pathogenicity testing, performed by detached leaf bioassay, were screened further for their damage symptoms and pathogenicity to waterhyacinth (**Table 3.1**) by whole

plant bioassay. A significant difference in the values of disease intensity in different hours of exposure of isolates of fungal inoculum on the waterhyacinth leaves (F_{3404}, $P<0.05$) was observed (**Table 3.2**). The symptoms of disease appearance (**Table 3.1**) started within 24 to 72 hours of application of the respective fungal spore and/or mycelial suspension, depending on the pathogenic potency of the fungi. There was a significant difference in the disease intensity caused by different isolates at 24 hours ($F_{(48, 245)} = 140.15$, $P<0.0001$), 48 hours ($F_{(48, 245)} = 118.35$, $P<0.0001$), 72 hours ($F_{(48, 245)} = 188.77$, $P<0.0001$), 96 hours ($F_{(48, 245)} = 231.83$, $P<0.0001$) and 120 hours ($F_{(48, 245)} = 180.13$, $P<0.0001$) after application of fungal inoculum on the waterhyacinth leaves.

Among *Alternaria* sps., WHK-34 isolate have left a significant effect within 24 hours of application, causing about 29.33 ± 3.05 % damage to the host, apart from *Fusarium* isolates WHK-59, WHK-106 and WHK-74 leaving 28.33 ± 2.89 %, 31.67 ± 5.73 % and 25.00 ± 5.00 % disease symptoms, respectively. After 48 hours, the effect of WHK-59 and 106 speeded up (to 68.67 ± 3.21 % and 71.33 ± 4.16 %, respectively) with their disease-causing severity. When the later spoken isolates were quick and consistent in their disease severity, some isolates like *P. salina* (WHK-37), *C. geniculatus* (WHK-23), *P. herbarum* (WHK-137), *S. rostrata* (WHK-96), and *A. hisapnicum* (WHK-104) showed its potency as a biocontrol agent against waterhyacinth, but started showing a significant effect (41.67 - 53.00 %) almost 72 hours from the date of application (**Figure 3.8**). However, apart from the tested isolates, the latter was comparatively effective in causing gradual sinking of the plant with an effective 70.00 - 96.33 % damage within 120 hours of inoculation.

The top 20 isolates (designated with # in **Table 3.1**) having the disease index of over 50 % of the total area of application, further underwent whole-plant bioassay, which were identified and characterized using molecular techniques (as mentioned in **Chapter 2**). Several *Alternaria* spp, and *Fusarium* spp. were found to be highly effective fungi, among them, seven fungal isolates including *A. alternata* (WHK-3, 38 and 42), *C. geniculatus* (WHK-36), *F. oxysporum* (WHK-59 and 106) and *P. salina* (WHK-37), caused disease intensity of more than 90 % (**Figure 3.8, Table 3.1**). Based on the disease initiation and intensity of infection, four fungi (**Figure 3.9**), *Alternaria alternata* (AA), *Fusarium oxysporum* sp. *lycopersici* 4287 (FO), *Fusarium verticillioides* (FR), *Paradendryphiella salina* (PS), were selected for the next round

of experiment for host-specificity, arthropod-pathogen interactive study and understanding their nature (**Table 3.3**). AA and FO showed maximum pathogenicity among the four intense phytopathogens.

TABLE 3.1: Phytopathogenic fungi and their symptoms of disease appearance

Isolate No.	Fungal Isolate	Damage Percentage (%) @	Symptom of disease
WHK-17	*Acremonium* sp.	34.00	Zonate leaf spots
WHK-3#	*Alternaria alternata* (Fr.) Keissler	65.67	Leaf spots
WHK-34#	*Alternaria alternata* (Fr.) Keissler	95.00	Leaf spot and blight
WHK-38#	*Alternaria alternata* (Fr.) Keissler	95.00	Brownish leaf spots
WHK-42#	*Alternaria alternata* (Fr.) Keissler	91.67	Leaf spot and blight
WHK-43#	*Alternaria alternata* (Fr.) Keissler	89.00	Scattered leaf spots
WHK-141#	*Alternaria alternata* (Fr.) Keissler	66.67	Leaf spots
WHK-12#	*Alternaria japonica* Yoshii 1941 *	66.67	Leaf spots
WHK-7	*Alternaria* sp.	31.67	Leaf spots
WHK-46	*Alternaria* sp.	20.00	Leaf spots
WHK-104#	*Arthrinium hispanicum* Larrondo & Calvo *	70.00	Leaf spots
WHK-89	*Cercospora* sp.	2.00	Leaf spots
WHK-10	*Cladosporium* sp.	2.70	No symptoms
WHK-21	*Cladosporium* sp.	1.97	No symptoms
WHK-24	*Cladosporium* sp.	2.20	No symptoms
WHK-103	*Cladosporium* sp.	2.33	No symptoms
WHK-135	*Cladosporium* sp.	2.83	No symptoms
WHK-1#	*Cochliobolus geniculatus* R.R. Nelson *	79.00	Leaf blight and spot blotch
WHK-23#	*Cochliobolus geniculatus* R.R. Nelson *	96.33	Leaf spot blotch
WHK-25	*Curvularia* sp.	2.50	Small necrotic spots
WHK-27	*Curvularia* sp.	1.20	Small necrotic spots
WHK-28	*Curvularia* sp.	2.50	Small necrotic spots
WHK-93	*Curvularia* sp.	2.00	Small necrotic spots
WHK-94	*Curvularia* sp.	2.30	Small necrotic spots
WHK-101	*Curvularia* sp.	2.17	Small necrotic spots
WHK-26	*Fusarium oxysporum (Schlecht)*	38.33	Irregular marginal spots
WHK-59#	*Fusarium oxysporum* sp. *lycopersici* *	91.67	Leaf necrosis

Isolate No.	Fungal Isolate	Damage Percentage (%) @	Symptom of disease
WHK-106#	*Fusarium oxysporum f* sp. *cumini* strain F11 *	93.33	Leaf necrosis
WHK-71	*Fusarium roseum* Link	31.67	Leaf blight
WHK-9	*Fusarium ventricosum* Appel & Wollenweber, *	27.33	Zonate leaf spots
WHK-74#	*Fusarium verticillioides* (Sacc.) Nirenberg *	71.67	Leaf spot and blight
WHK-87#	*Fusarium verticillioides* (Sacc.) Nirenberg *	61.67	No symptom
WHK-13	*Fusarium* sp.	12.33	Leaf spots
WHK-88	*Fusarium* sp.	9.00	Central dark brown spots
WHK-92	*Fusarium* sp.	14.00	Leaf spots
WHK-99	*Fusarium* sp.	16.33	Leaf spots
WHK-136	*Fusarium* sp.	26.33	Leaf spots
WHK-30	*Myrothecium* sp.	7.50	Leaf lesions
WHK-142	*Nigrospora* sp.	1.20	Leaf blight
WHK-37#	*Paradendryphiella salina* (G.K. Sutherland) Woudenberg & Crous *	92.00	Chlorotic patches
WHK-97#	*Pleospora herbarum* (Pers.) Rabenh.	64.00	Oval yellow spots
WHK-137#	*Pleospora herbarum* (Pers.) Rabenh.	78.33	Oval yellow spots
WHK-138#	*Pleospora herbarum* (Pers.) Rabenh.	61.67	Oval spots
WHK-20	*Rhizoctonia* sp.	37.67	Leaf blight and spots
WHK-96#	*Setosphaeria rostrata* K.J. Leonard *	70.00	Leaf spots
WHK-152	*Stemphylium* sp.	37.33	Leaf spots
WHK-19	*Trichothecium* sp.	46.67	No symptoms
WHK-36#	*Ulocladium botrytis* Preuss	58.33	Leaf spots

Disease index of over 50 % of the total area of application (after 120 hours of application)

* These isolates were recorded for the first time from waterhyacinth

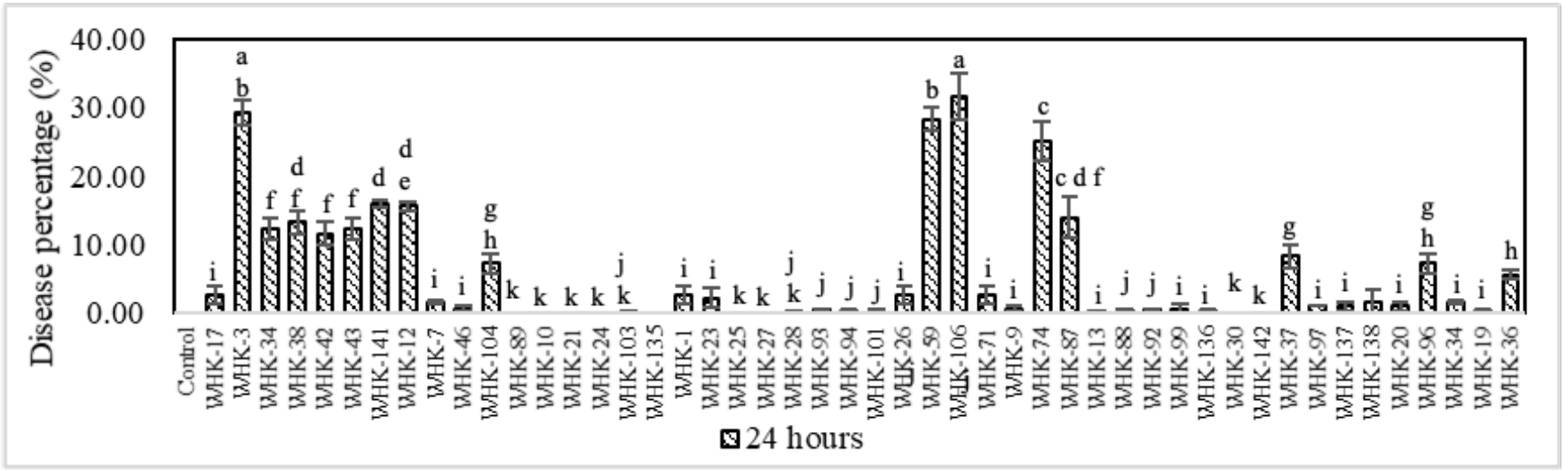
@ The damage percentage represented are after 120 hours of inoculation

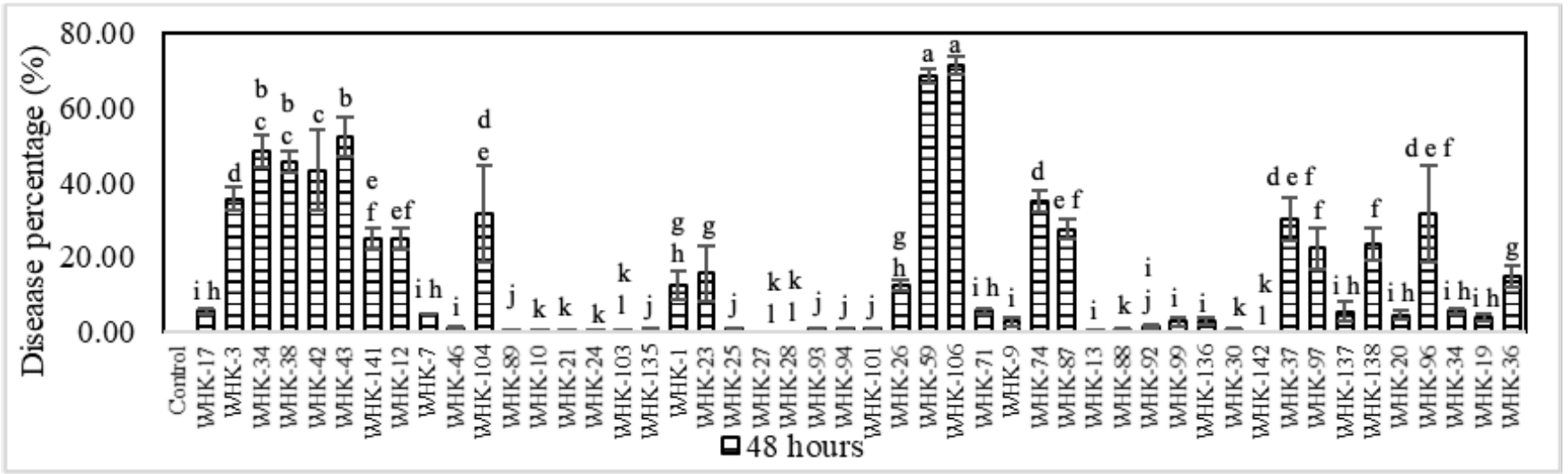
Table 3.2: Repeated measures of ANOVA results for various isolates in different time points (24, 48, 72, 96 and 120 hours).

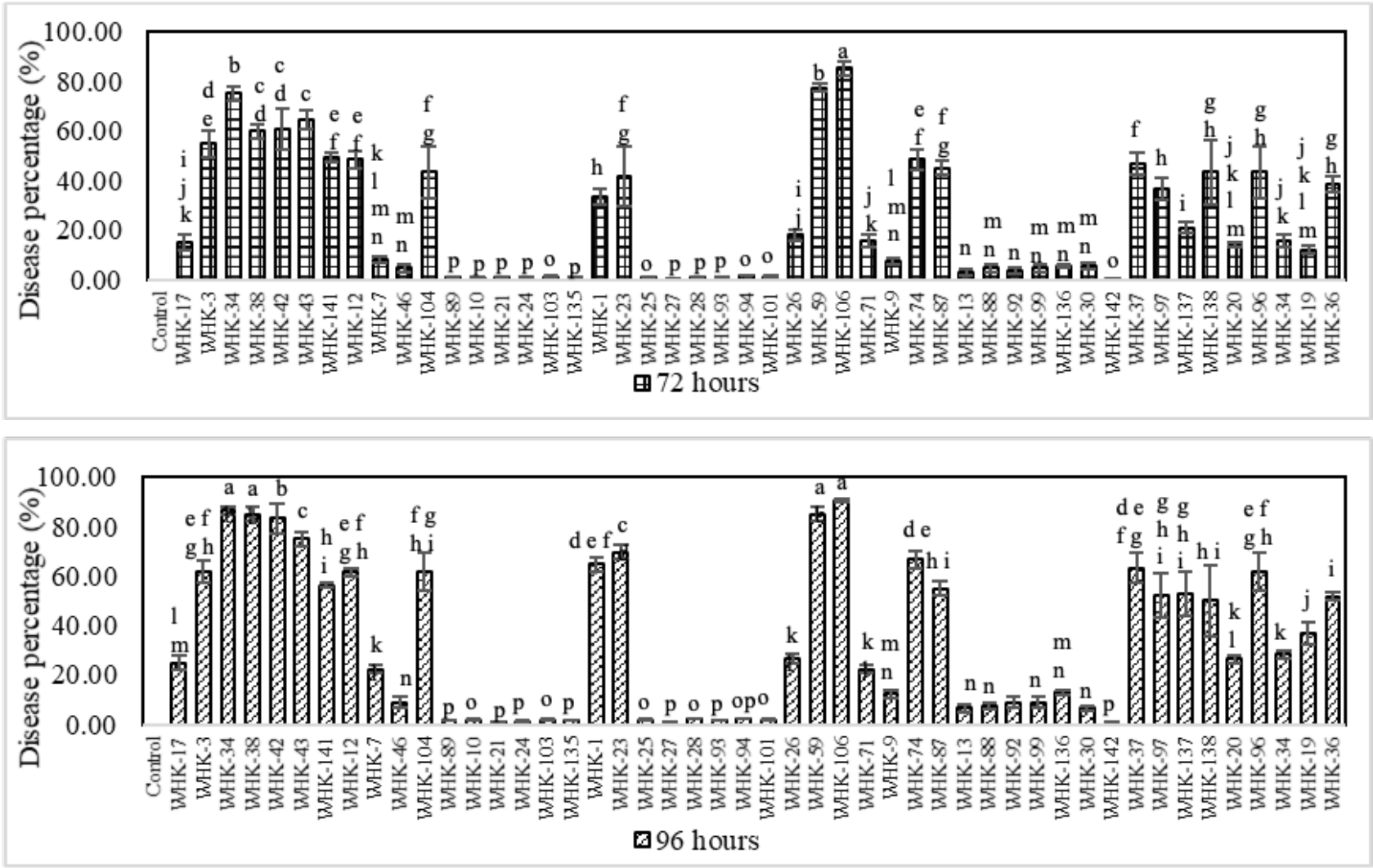
Tests of Within-Subjects Effects						
Measure: Disease Density						
Source		Type III Sum of Squares	df	Mean Square	F	Sig.
Hrs	Sphericity Assumed	314785.288	4	78696.322	3404.267	.000
	Greenhouse-Geisser	314785.288	3.161	99581.685	3404.267	.000
Hrs * Isolates	Sphericity Assumed	80223.210	120	668.527	28.919	.000
	Greenhouse-Geisser	80223.210	94.832	845.948	28.919	.000

Table 3.3: Pathogenicity of the four potent fungi on waterhyacinth

Sl. No.	Pathogens	Treatment	Disease percentages {Mean (±SD)}		
			Day 1	**Day 3**	**Day 5**
1	***Alternaria alternata***	Scratched	16.67 (±2.89)	65.00 (±13.23)	83.33 (±15.28)
		Unscratched	4.67 (±2.52)	43.33 (±10.41)	73.33 (±15.28)
2	***Fusarium oxysporum***	Scratched	20.25 (±9.07)	61 (±25.06)	85 (±23.61)
		Unscratched	12 (±2.89)	45.67 (±14.58)	76.67 (±15.81)
3	***Fusarium verticillioides***	Scratched	12.33 (±9.29)	50.00 (±10.00)	66.67 (±15.28)
		Unscratched	2.67 (±2.08)	42.00 (±13.11)	66.67 (±5.77)
4	***Paradendryphiella salina***	Scratched	0.54 (±0.10)	23.6 (±10.41)	67 (±12.42)
		Unscratched	0.34 (±0.15)	23.20 (±5.75)	71.4 (±4.43)
5	**Control**	Scratched	0.00	0.00	0.00
		Unscratched	0.00	0.00	0.00


Disease percentage (%)
40.00
30.00
20.00
10.00
0.00
Control
WHK-17
WHK-3
WHK-34
WHK-38
WHK-42
WHK-43
WHK-141
WHK-12
WHK-7
WHK-46
WHK-104
WHK-89
WHK-10
WHK-21
WHK-24
WHK-103
WHK-135
WHK-1
WHK-23
WHK-25
WHK-27
WHK-28
WHK-93
WHK-94
WHK-101
WHK-26
WHK-59
WHK-106
WHK-71
WHK-9
WHK-74
WHK-87
WHK-13
WHK-88
WHK-92
WHK-99
WHK-136
WHK-30
WHK-142
WHK-37
WHK-97
WHK-137
WHK-138
WHK-20
WHK-96
WHK-34
WHK-19
WHK-36
24 hours


Diseaase percentage (%)
80.00
60.00
40.00
20.00
0.00
Control
WHK-17
WHK-3
WHK-34
WHK-38
WHK-42
WHK-43
WHK-141
WHK-12
WHK-7
WHK-46
WHK-104
WHK-89
WHK-10
WHK-21
WHK-24
WHK-103
WHK-135
WHK-1
WHK-23
WHK-25
WHK-27
WHK-28
WHK-93
WHK-94
WHK-101
WHK-26
WHK-59
WHK-106
WHK-71
WHK-9
WHK-74
WHK-87
WHK-13
WHK-88
WHK-92
WHK-99
WHK-136
WHK-30
WHK-142
WHK-37
WHK-97
WHK-137
WHK-138
WHK-20
WHK-96
WHK-34
WHK-19
WHK-36
48 hours


Disease percentage (%)
100.00
80.00
60.00
40.00
20.00
0.00
Control
WHK-17
WHK-3
WHK-34
WHK-38
WHK-42
WHK-43
WHK-141
WHK-12
WHK-7
WHK-46
WHK-104
WHK-89
WHK-10
WHK-21
WHK-24
WHK-103
WHK-135
WHK-1
WHK-23
WHK-25
WHK-27
WHK-28
WHK-93
WHK-94
WHK-101
WHK-26
WHK-59
WHK-106
WHK-71
WHK-9
WHK-74
WHK-87
WHK-13
WHK-88
WHK-92
WHK-99
WHK-136
WHK-30
WHK-142
WHK-37
WHK-97
WHK-137
WHK-138
WHK-20
WHK-96
WHK-34
WHK-19
WHK-36
72 hours
Disease percentage (%)
100.00
80.00
60.00
40.00
20.00
0.00
Control
WHK-17
WHK-3
WHK-34
WHK-38
WHK-42
WHK-43
WHK-141
WHK-12
WHK-7
WHK-46
WHK-104
WHK-89
WHK-10
WHK-21
WHK-24
WHK-103
WHK-135
WHK-1
WHK-23
WHK-25
WHK-27
WHK-28
WHK-93
WHK-94
WHK-101
WHK-26
WHK-59
WHK-106
WHK-71
WHK-9
WHK-74
WHK-87
WHK-13
WHK-88
WHK-92
WHK-99
WHK-136
WHK-30
WHK-142
WHK-37
WHK-97
WHK-137
WHK-138
WHK-20
WHK-96
WHK-34
WHK-19
WHK-36
96 hours

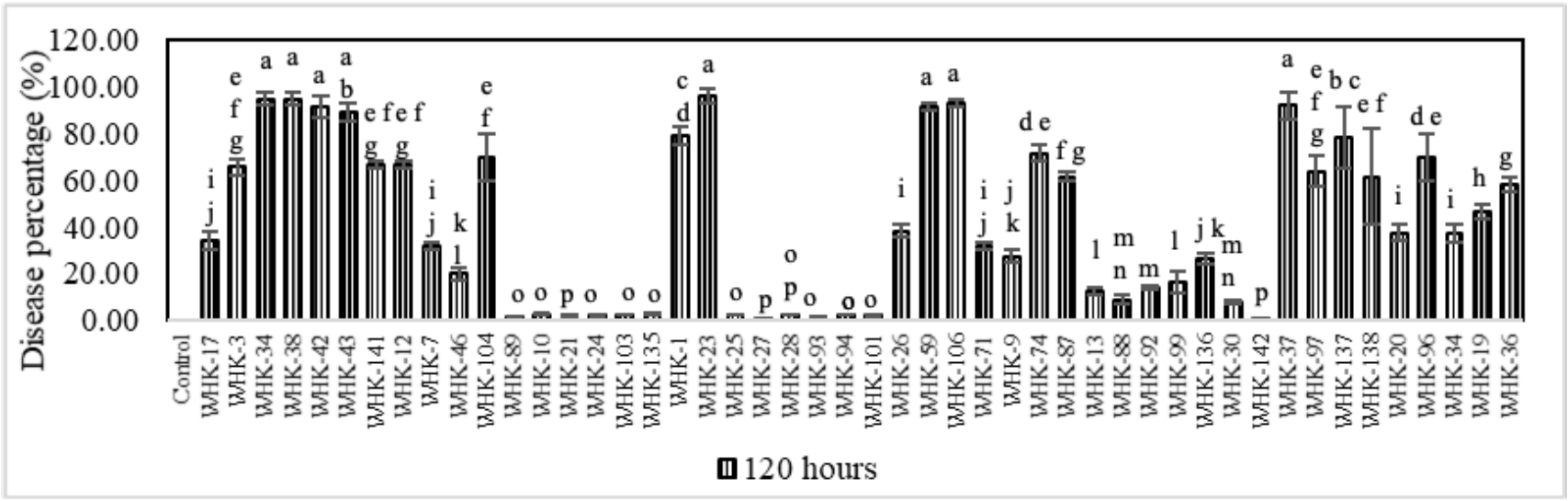


Figure 3.8: Disease percentage of various isolates of fungi, on waterhyacinth after (i) 24 hours (ii) 48 hours (iii) 72 hours (iv) 96 hours and (v) 120 hours. (Detached leaf bioassay)

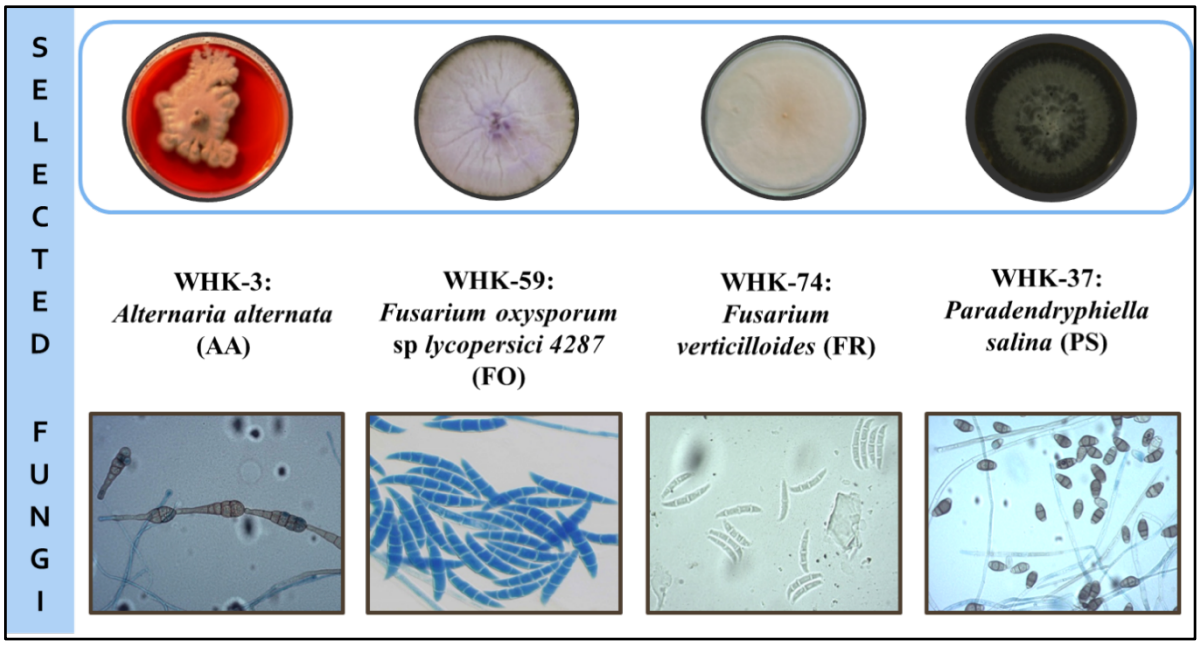


Figure 3.9: The four potent phytopathogens, among the array of phytopathogens, found effective against the host, waterhyacinth, for their pathogenicity and biocontrol potential

3.3.2. *Host-range specificity test*

The above-mentioned four potent fungal isolates including AA, FO, FR and PS were selected for host range studies (**Table 3.4**). The spore suspension and culture filtrate of the four species were effective against their host, waterhyacinth.

Apart from that, the culture filtrate of AA showed its effect mostly on other weeds like *Pistia stratiotes, Alternanthera sessilis, Trianthema portulascastrum, Sinapis alba, Ipomoea* aquatic, *Hydrilla verticillata* and *Rumex obtusifolius*, and two cultivated crops like *Spinacia oleracea* and *Chenopodium album*. The spore suspension of AA showed little effects on *C. album*, apart from the host itself. PS had shown its effect at a much broader spectrum, effecting almost all the plants that AA infected and apart from them a few more. In the additional list of infected plants *A. philoxeroides, Amaranthus spinosus, Blumea lacera, Tridax procumbens, Ceratophyllum demersum* all are weeds while *Lycopersicon esculentum* is a cultivated plant. *A. philoxeroides, H. verticillata* and *P. stratiotes* are ecologically related to the host, waterhyacinth. When the culture filtrate is so effective and broad-ranged, the spore suspension of PS is highly host-specific. FO has shown its minimum effect on the two

cultivated plants (*S. oleracea* and *C. album*) and a weed (*R. obtusifolius*). The spore suspension of FR has been limited to two aquatic weeds-waterhyacinth and *A. philoxeroides*, while the culture filtrate has shown effects on three out of four *Alternanthera* sps., used for testing, apart from another weed, *Trianthema portulacastrum*.

Table 3.4: Host-range testing of selected fungal pathogens on various crops and weed hosts

S.no.	Family	Botanical name (Common name/Vernacular name in India)	AA		FO		FR		PS	
			s	t	s	t	s	t	s	t
1.	Aizoaceae	*Trianthema portulacastrum* L.[c] (Horse-purslane/ Pathar chata)	-	+	-	-	-	+	-	+
2.	Amaranthaceae	*Alternanthera philoxeroides* (Mart.) Griseb. [b c] (Alligator weed/ Pani-khutura)	-	-	-	-	+	+	-	-
3.		*Alternanthera brasiliana* (L.) Kuntze. [c] Brazilian joyweed	-	-	-	-	-	+	-	-
4.		*Alternanthera pungens* H.B & K. [c] khaki weed	-	-	-	-	-	-	-	-
5.		*Alternanthera sessilis* L. [c] (Sessile joyweed/ Kantewali santhi)	-	-	-	-	-	+	-	+
6.		*Amaranthus viridis* L. [a] (Amaranth/ Chaulai)	-	+	-	-	-	-	-	+
7.		*Amaranthus tricolor* L. [c] *(Tricolor amaranth)*	-	-	-	-	-	-	-	-
8.		*Amaranthus spinosus* L. [c] (Spiny pigweed)	-	-	-	-	-	-	-	+
9.		*Digera arvensis* Forssk [c] *(False amaranth)*	-	-	-	-	-	-	-	-
10.		*Celosia argentea* L [c] *c (Cock's comb)*	-	-	-	-	-	-	-	-
11.	Apiaceae	*Centella asiatica* L. [c] (Asian pennywort/ Brahmi)	-	-	-	-	-	-	-	-
12.	Araceae	*Pistia stratiotes* L. [b c] (Water lettuce)	-	+	-	-	-		-	+
13.		*Colocasia esculenta* (L.) Schott [c] (Taro)	-	-	-	-	-	-	-	-

S.no.	Family	Botanical name (Common name/Vernacular name in India)	AA		FO		FR		PS	
			s	t	s	t	s	t	s	t
14.	Asteraceae	*Ageratum conyzoides* L. [c] Goat weed	-	-	-	-	-	-	-	-
15.		*Blumea lacera* DC [c] (False oxtongue/ Kukurbanda)	-	-	-	-	-	-	-	+
16.		*Cichorium intybus* L. [c] (Chickory/ Kasani)	-	-	-	-	-	-	-	-
17.		*Parthenium hysterophorus* L. [c] (Parthenium/ Gajar ghas)	-	-	-	-	-	-	-	-
18.		*Sonchus arvensis* L. [c] (Perennial sowthistle/ Bhatkataiya)	-	-	-	-	-	-	-	-
19.		*Tagetes erecta* L. [a] (Marigold/ Genda)	-	-	-	-	-	-	-	-
20.		*Tridax procumbens* L. [c] (Coat buttons/ Phulani)	-	-	-	-	-	-	-	+
21.		*Vernonia cinerea* [c] (Little ironweed)	-	-	-	-	-	-	-	-
22.	Asclepiadiaceae	*Calotropis gigantea* [c] (Giant milkweed)	-	-	-	-	-	-	-	-
23.	Azollaceae	*Azolla pinnata* Brown [bc] (Feathered mosquitofern)	-	-	-	-	-	-	-	-
24.	Boraginaceae	*Heliotropium indicum* L. [c] (Indian heliotrope)	-	-	-	-	-	-	-	-
25.	Brassicaceae	*Brassica campestris* L. *var sarson* [a] (Rai/ Sarson)	-	-	-	-	-	-	-	-
26.		*Raphanus sativus* L. [a] (Radish/ Mooli)	-	-	-	-	-	-	-	-
27.		*B. oleracea* L. *var. botrytis* [a] (Cauliflower/ Phool gobhi)	-	-	-	-	-	-	-	-
28.		*B. oleracea* L. *var. capitata* [a]	-	-	-	-	-	-	-	-

S.no.	Family	Botanical name (Common name/Vernacular name in India)	AA		FO		FR		PS	
			s	t	s	t	s	t	s	t
		(Cabbage/ Bandha gobhi)								
29.		*Sinapis alba* L. [c] (Wild mustard/ Safed Rai)	-	+	-	-	-	-	-	-
30.	Capparidaceae	*Cleome viscosa* L.[c] (Asian spider flower)	-	-	-	-	-	-	-	-
31.	Caricaceae	*Carica papaya* L. [a] (papaya)	-	-	-	-	-	-	-	-
32.	Ceratophyllaceae	*Ceratophyllum demersum* L[b c] (hornwort, coontail)	-	-	-	-	-	-	-	+
33.	Chenopodiaceae	*Chenopodium album* L. [a c] (Goosefoot/ Bathua)	+	+	-	+	-	-	-	-
34.		*Spinacia oleracea* L. [a] (Spinach/ Palak)	-	+	-	+	-	-	-	+
35.	Commelinaceae	*Commelina benghalensis* L. [c] (Tropical Spiderwort/ Kanteri)	-	-	-	-	-	-	-	-
36.		*Cyanotis axillaris* (L.) D.Don ex Sweet [c] (Creeping Cradle Plant)	-	-	-	-	-	-	-	-
37.	Convolvulaceae	*Convolvulus arvensis* L. [c] (Bindweed/ Hiran chara)	-	-	-	-	-	-	-	-
38.		*Ipomoea fistulosa* Mart. [c] (Morning Glory/ Beshram)	-	-	-	-	-	-	-	-
39.		*Ipomoea aquatic* Forsk[b c] (Water spinach/ Kalmi sag)	-	+	-	-	-	-	-	-
40.	Cyperaceae	*Cyperus iria* L. [c] (Rice foot sedge/ Galmotha)	-	-	-	-	-	-	-	-
41.	Euphorbiaceae	*Euphorbia hirta* L. [c] (Asthma weed/ Dudhi)	-	-	-	-	-	-	-	-
42.	Fabaceae	*Cicer arietinum* L. [a] (Gram/ Chana)	-	-	-	-	-	-	-	-

S.no.	Family	Botanical name (Common name/Vernacular name in India)	AA		FO		FR		PS	
			s	t	s	t	s	t	s	t
43.		*Glycine max* L. [a] (Soybean)	-	-	-	-	-	-	-	-
44.		*Lens esculenta* Moench [a] (Lentil/ Masoor)	-	-	-	-	-	-	-	-
45.		*Medicago polymorpha* L. [c] (Medick)	-	-	-	-	-	-	-	-
46.		*Pisum sativum* L. [a] (Pea/ Matar)	-	-	-	-	-	-	-	-
47.		*Trifolium alexandrium* L. [c] (Egyptian clove/ Barseem)	-	-	-	-	-	-	-	-
48.		*Vigna radiata* L. [a] (Mung bean/ Moong)	-	-	-	-	-	-	-	-
49.	Gramineae	*Brachiaria mutica* (Forsk.) Stapf. [c] (Para grass)	-	-	-	-	-	-	-	-
50.		*Cynodon dactylon* L. [c] (Bermuda grass/ Dubh)	-	-	-	-	-	-	-	-
51.		*Oryza sativa* L. [a] (Paddy/ Dhan)	-	-	-	-	-	-	-	-
52.		*Triticum aestivum* L. [a] (Wheat/ Gehoon)	-	-	-	-	-	-	-	-
53.		*Zea mays* L. [a] (Maize, Corn/ Bhutta)	-	-	-	-	-	-	-	-
54.	Hydrocharitaceae	*Hydrilla verticillata* (L. f.) Royle [b c] (Hydrilla)	-	+	-	-	-	-	-	-
55.	Lamiaceae	*Hyptis suaveolens* L. Point. [c] (Pignut/ Wilayati tulsi)	-	-	-	-	-	-	-	-
56.	Lemnaceae	*Lemna minor* L. [b c] (Common duckweed)	-	-	-	-	-	-	-	-
57.	Linaceae	*Linum usitatissimum* L. [a]	-	-	-	-	-	-	-	-

S.no.	Family	Botanical name (Common name/Vernacular name in India)	AA		FO		FR		PS	
			s	t	s	t	s	t	s	t
		(Linseed/ Alsi)								
58.	Malvaceae	*Sida acuta* Burm. f. [c] (Common wire weed/ Kareta)	-	-	-	-	-	-	-	-
59.		*Gossypium hirsutum* [a] (Cotton, non bt)	-	-	-	-	-	-	-	-
60.	Marsileaceae	*Marsilea minuta* L. [bc] (Pepperwort)	-	-	-	-	-	-	-	-
61.	Polygonaceae	*Rumex obtusifolius* L. [c] (Broad-leaved dock/ Jungli palak)	-	+	-	+	-	-	-	+
62.	Pontederiaceae	*Eichhornia crassipes* (Mart.) Solms (Waterhyacinth/ Jal kumbhi)	+	+	+	+	+	-	+	+
63.		*Monochoria vaginalis* [bc] (Pickerel weed)	-	-	-	-	-	-	-	-
64.	Solanaceae	*Lycopersicon esculentum* Mill. [a] (Tomato/ Tamaatar)	-	-	-	-	-	-	-	+
65.		*Physalis minima* L. [c] (Wild gooseberry/ Pachkotta)	-	-	-	-	-	-	-	-
66.	Trapaceae	*Trapa natans* L. [b] (Water chestnut)	-	-	-	-	-	-	-	-
67.	Verbenaceae	*Lantana camara L.* [c] (*Lantana/ Makoiya)*	-	-	-	-	-	-	-	-

[a] Cultivated plant
[b] Plant ecologically related to the test plant
[c] Non-native weed
[d] Plant reported susceptible to cultivars of *A. japonica*
***** Spore suspension was sprayed in water containing hydrilla while phytotoxicity was accessed by growing the hydrilla shoot in the culture filtrate.
+ damage caused, - no damage
s- Spore suspension (5 x 10^5 spores/ml)
t- Culture filtrate

3.3.3. *Arthropod- plant-pathogen interaction studies*

Biocontrol agents in various combination had cumulative impact on waterhyacinth. The development of damage caused by these agents, singly or in combinations, were recorded as damage percentage. Disease instigated by phytopathogens were visually manifested in the form of necrotic patches or chlorotic spots, whereas the arthropods had their characteristic feeding marks. In the arthropod-plant-pathogen interaction study (**Figure 3.10**), initially within 24 hours, each fungus alone or in combinations with arthropods had their own rate and intensity of causing damage.

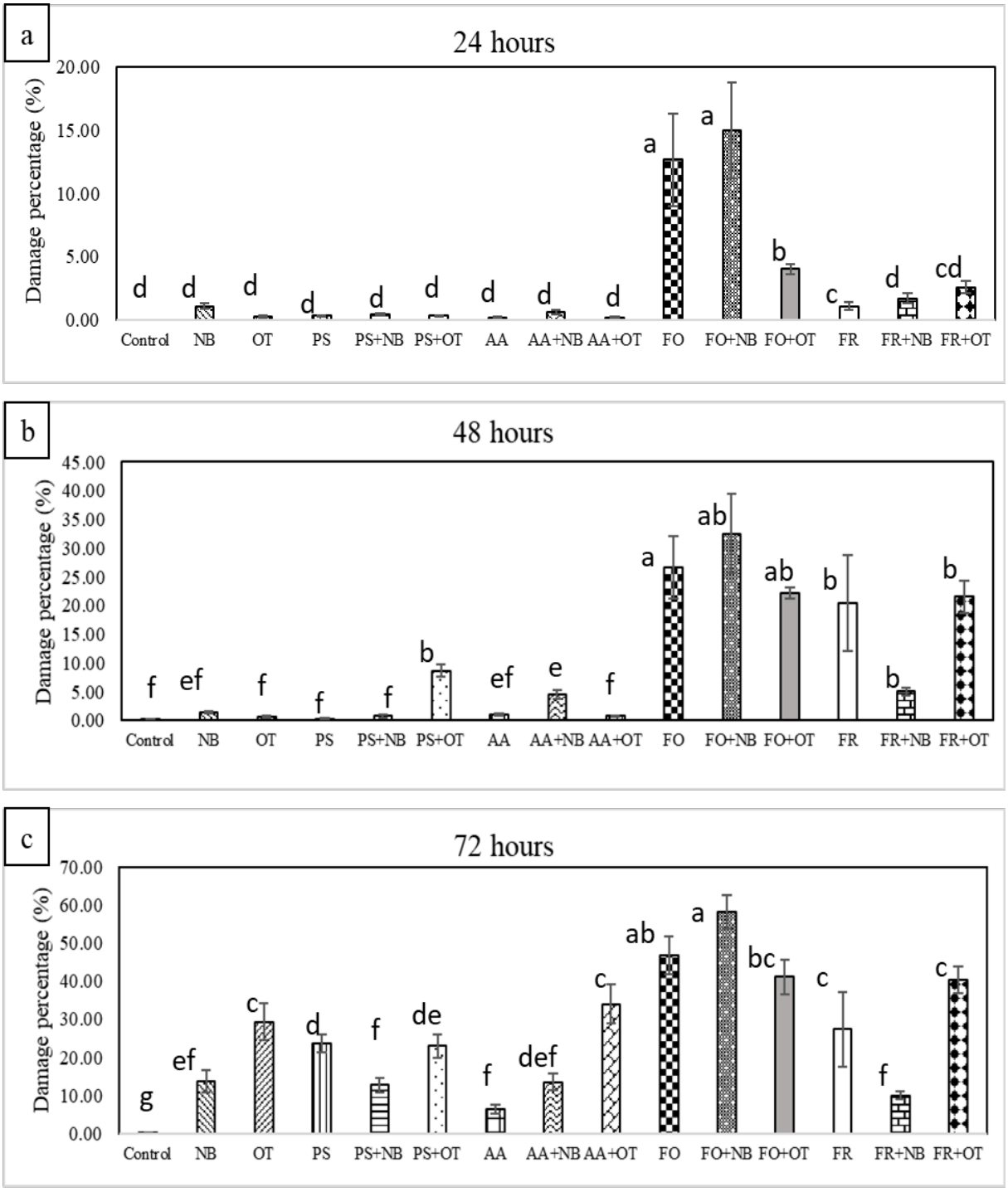
FO suspension when applied singly showed quick effect, mainifested with disease symptom initiation within 3 hours of application, with prominent necrotic spots appearing after 24 hours with 13 % ± 3.9 damage appearing on waterhyacinth. With the advancement of hours, the infection rate inclined to 81.3 % ± 5.2 of damage within 120 hours of inoculation, leading to the decaying, drowning and eventually death of the plant. FO when combined with other agents, showed significantly greater damage on waterhyacinth as compared to other treatments, with maximum damage percentage shown by FO + NB combination. FO + NB, showed a rise in the damage percentage, in comparison to when applied alone (FO). Delayed disease-initiation with 4.0 % ± 0.4 necrosis was observed in FO + OT treated plant while FO + NB had a comparatively high damage with 15.0 % after 24 hours ($F_{14,75}$=6.67, P=0.00), and enhanced damage percentage of about 89.80 % ± 2.8, by 120 hours.

PS treated plants showed disease initiation only after 72 hours. PS, when applied singly, showed its characteristic chlorotic spots. It caused death of the weed by decreasing the chlorophyll content of the plant and hampering the basic essential function for growth. PS appeared as a slow disease initiating pathogen in comparison to the others agents applied, since it was not able to show any significant chlorosis 48 hours (0.34 % ± 0.1) after the treatment, neither when applied singly, nor in any arthropodal combinations (PS + NB and PS + OT). PS + OT caused 73.2 % ± 3.0 by 120 hours of application of the biocontrol agents. PS + NB, on the other hand, showed a disease percentage was 0.44 % ± 0.1 only, which was slightly more than when applied singly. PS had a repulsive effect with weevils (NB) causing 55.8 % ± 4.7 damage after 120 hours ($F_{14, 75}$ = 22.13, P=0.00), with weevils' death and delayed disease initiation. The combined effect of PS+OT was 23 %, almost close to the individual effect of PS

(23.7 %), while PS+NB interaction resulted even lower damage of 12.7 %, after 72 hours. The weevils could not be traced in the PS + NB treatments, within 24 hours of application. By 72 hours, drowned, dead and immotile weevils were noticed in large numbers in many pots.

FR when applied singly caused 9.4 % ± 4.2 damage by 24 hours. FR showed initially disease manifestation with necrosis, then slowly showing chlorotic spots along the margins of necrotic areas. Damage percentage was higher for FR + OT treatment with 94.3 % ± 1.2 as compared to FR alone (63.3 % ± 12.2), completely opposite to the effect on being applied with weevils (FR + NB) with 32.7 %, after 120 hours, which being 1.7 % ± 0.4, after 24 hours of treatment. It hardly caused 35 % damage after 5 days in the cumulative action. The damage intensity was proportionately very high and facilitated in case of FR + OT treatment. An effective damage of about 2.6 % ± 0.5 was observed after 24 hours, while it showed damage percentage of 94.3 % ± 1.2 after the 5^{th} day in comparison to 63.3 % ± 12.2 when the fungi was applied singly.

AA when applied singly, started to show its effect after 72 hours of treatment ($F_{14, 75}$=19.30, P=0.00). The cumulative impact of AA + OT and AA + NB were much higher with respect to effect of the phytopathogen, single-handedly. Though AA + NB showed a rise of 13.5 % ± 2.1 within 48 hours of treatment, it was found that AA + OT had a higher impact on damage causing about 34 % ± 5.2 of damage on the host plant, after 72 hours of treatment. The damage percentage tolled up to 89.75 % ± 1.13 for AA + OT at the end of 120 hours, while AA and AA + NB showed about 50 % of the total damage. The damage of AA was purely necrotic, with the weevils leaving their feeding scars and the mite was found clumped near to their feeding places. Lack of chlorosis was prominent, as the leaves turned almost dark brown to black in colour, prior to their death.


a
24 hours
Damage percentage (%)
20.00
15.00
10.00
5.00
0.00
d d d d d d d d d a a b c d cd
Control NB OT PS PS+NB PS+OT AA AA+NB AA+OT FO FO+NB FO+OT FR FR+NB FR+OT
b
48 hours
Damage percentage (%)
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
f ef f f f b ef e f a ab ab b b
Control NB OT PS PS+NB PS+OT AA AA+NB AA+OT FO FO+NB FO+OT FR FR+NB FR+OT
c
72 hours
Damage percentage (%)
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
g ef c d f de f def c ab a bc c f c
Control NB OT PS PS+NB PS+OT AA AA+NB AA+OT FO FO+NB FO+OT FR FR+NB FR+OT

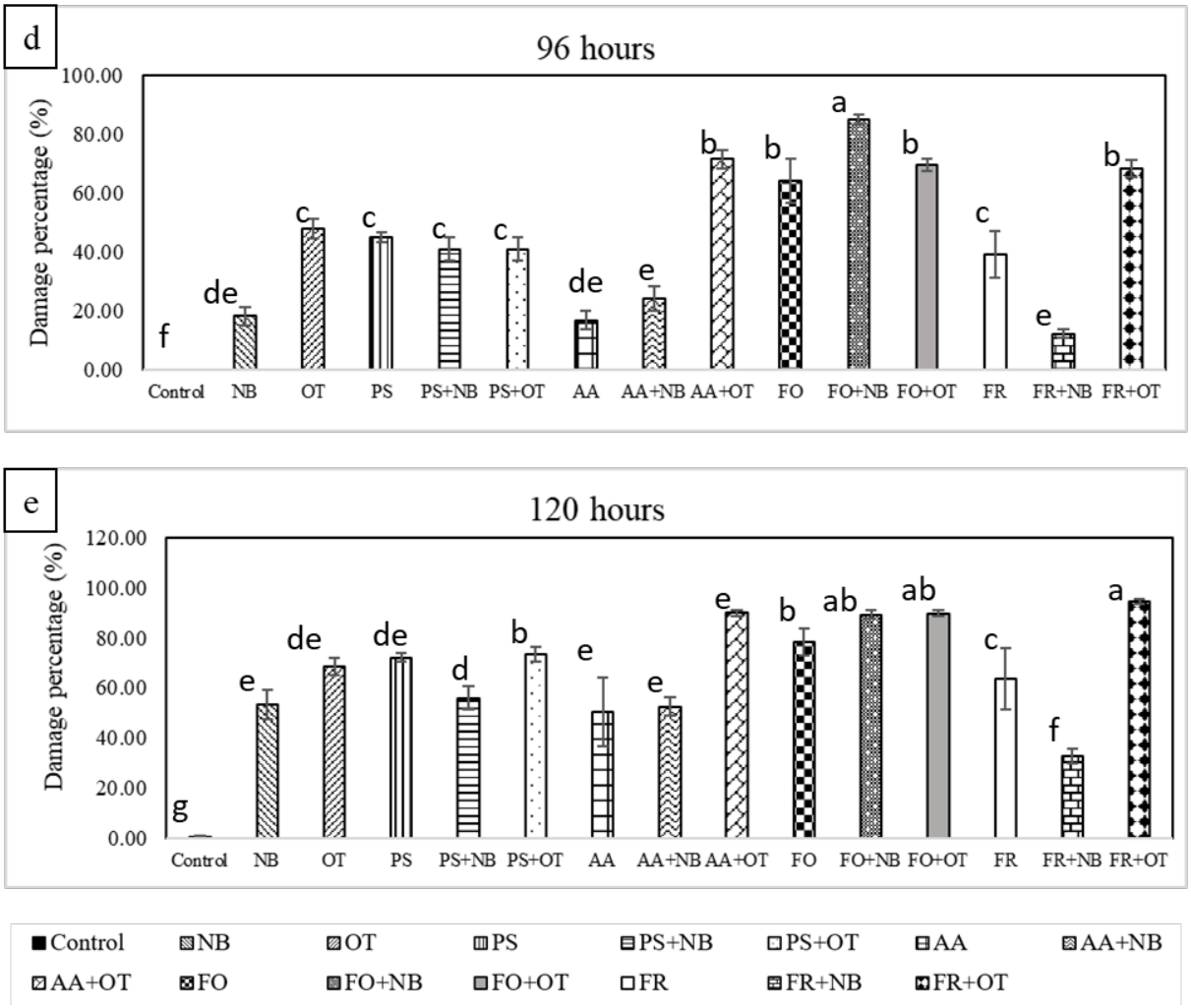


Figure 3.10: Arthropod-plant-pathogen interaction studies in various combinations at intervals of (a) 24 hours; (b) 72 hours; (c) 120 hours respectively. Mean bars having different alphabets are significantly different (P<0.05)

NB- *Neochetina bruchi* (chevroned waterhyacinth weevil)

OT- *Orthogalumna terebrantis* (waterhyacinth mite)

AA- *Alternaria alternata*

FO- *Fusarium oxysporum* sp. *lycopersici* 4287

FR- *Fusarium verticilloides*

PS- *Paradendryphiella salina*

3.3.3.1. **Host choice test**

Earlier PS + NB combination showed an exclusive observation where the weevils were found to be dead or vanished and no trace were found. Such negative interaction recorded in the PS + NB combination diverted in the performance of this second part of the study, where experiment was performed to observe the host choice of the weevils on PS treated and as well as fresh uninfected waterhyacinth plants. 12 hours after the initiation of the experiment, it was observed that all the weevils were actively feeding on the fresh plants, while no weevils were observed on the PS treated plants. Uninfected waterhyacinth showed 37 feeding marks from NB, while a complete absence of any fed mark was observed on the PS treated plant. After 24 hours, the 50 feeding scares was observed and after 120 hours, it was clearly seen that weevils had an fondness toward fresh uninfected waterhyacinth than PS treated ones ($F_{9,20}$ = 623.55, P < 0.0001) (**Figure 3.11**). An exponential rise in the NB feeding marks with the passage of time, ultimately caused breaking the rigidity of the test plant and drowning. On inavailability of fresh plants, or no fresh space on the lamina were available to feed on, fresh feeding marks were observed on the PS treated plants.

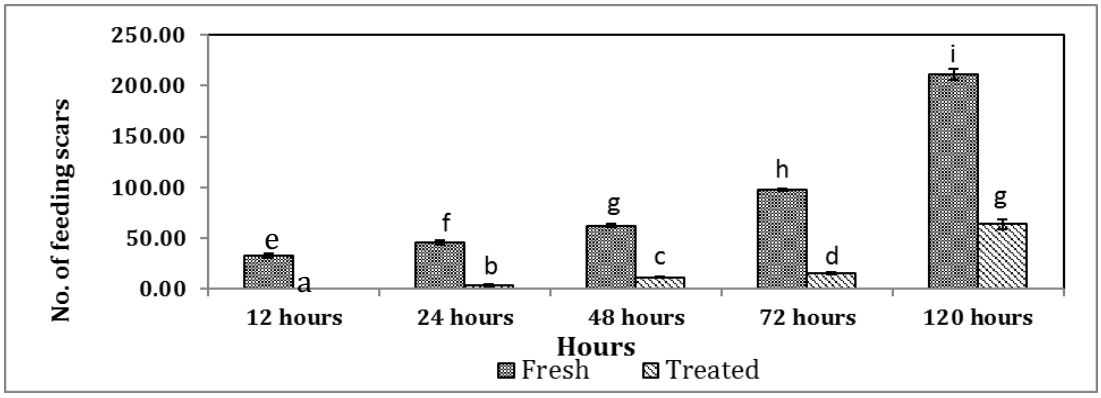


Figure 3.11: Feeding scars produced by *Neochetina* sp., on fresh (uninfected) waterhyacinth leaves versus *Paradendryphiella salina* spore-treated macrophyte. Mean marked by same letter(s) are not significantly different from each other (P = <0.0001) for all the graphs. Vertical bars denote indicate standard error

3.4. Discussion

Several pathogens have co-evolved and distributed themselves globally with the weed, waterhyacinth, itself (Ray and Hill 2012a). But the pathogenicity of these fungi varies from one isolate to another as recorded in the case of *F. oxysporum* by Praveena and Naseema (2006). Prevalence of *Fusarium* spp., followed by *Alternaria* spp., which is contrary to many survey reports conducted worldwide, where the widespread of the latter is much higher (Ray and Hill 2012a, Firehun et al. 2017), with a minimum of around 10 hours of incubation for the pathogen to show its effect in presence of a moist condition (Shabana et al. 1995a). Isolates of *A. alternata* has proved as an effective biocontrol agent around the globe, from Australia (Galbraith 1987) to Indian subcontinent (Bardur-ud-Din 1978, Aneja and Singh 1989, Babu et al. 2002) or against other weed species (Saxena and Pandey 2002). While some species of *Fusarium* and *Alternaria* have proved them to be one of the most promising biocontrol agents against the host, some have shown moderate to almost no effects after the pathogenicity trials, very similar to what Firehun and his colleagues had seen in his survey reported back to 2017 from Ethiopia (Firehun et al. 2017). Many isolates of *A. alternata* though had a very quick response against the weed; the autotoxic behaviour of the phytopathogens was the main reason for its short living (Saxena and Pandey 2002). Similar trends of quick response followed by a rapid decline in activity was shown by some isolates of *P. herbarum* (WHK-87) and *A. japonica* (WHK-12), which might indicate a favourable beginning with the availability of resources and their depletion with time along with autotoxicity of the respective phytopathogens (Singh and Rai 1981).

Several reports refraining to the fact that various strains of *A. alternata*, around the globe (Lau and Sheridan 1975, Fisher and Petrini 1992), are known to cause leaf blight disease in *O. sativa*, but WHK-3 strain of *A. alternata* has shown no such effect. Hence, the variant of this phytopathogen's susceptibility to rice, have been restricted for use in field conditions, as rice is one of the most cultivated crops in India and restricting the propagation of this fungal strain to other important crop plants will be difficult. Babu et al. (2003) states that host-specific toxins or culture filtrate are toxic to the host that are vulnerable to the fungi producing them. But, the culture filtrate of WHK-3 strain of *A. alternata* had a narrow range of effect on the plants belonging to Amaranthaceae family, unlike some previous reports (Sharma et al. 2013). Similarly,

whatever effect the WHK-59 isolates of *F. oxysporum* had on other economic and ecologically important, plants were mostly caused by the fungal phytotoxin. However, the phytotoxin producing isolate remains unaffected by their own toxin (Amusa 2006). Sometimes the cumulative effect of both the culture filtrate and toxin leads to the partial or complete death of the host (Wolpert et al. 1995). The damage caused by the phytotoxin sometimes gives an entry point, through the necrotic region, for spores of the same or other fungi to hasten the decomposition and degradation process (Wheeler and Luke 1963). *F. oxysporum* (WHK-59) with high effectiveness against waterhyacinth is substantially host specific hence used in our further studies.

Low damage potential single-handedly by phytopathogens alone have highlighted on the use of two or more agents together showing cumulative (Evans and Reeder 2001) for enhanced biocontrol avoiding antagonistic impact (Ray and Hill 2016, Dutta and Ray 2017). For the interactive studies among arthropods and phytopathogens, the four potent fungal inoculum (AA, FO, FR and PS) showed rigorous damaging effects against the macrophyte. FR + OT and FR + NB showed an enhanced impactful detrimentation on the weed, in reference to when the agents were applied individually. Multiple agents used against the target weed have shown successful outcomes in biological control than just single agent treatments (Hatcher 1995, Denoth et al. 2002), and such line of events was seen for FR + OT and FR + NB combinations. Such similar actions were cited by Kremer and Spencer (1989) where they mentioned about the infection caused by pathogenic fungi which enhanced the control of velvetleaf seed production with the scentless plant bug. Studies like these hence demonstrates that insect feeding enhances the rate of seed-borne pathogens in seed up to 98 % than just an average fungal infection of 8 % for seeds (Dutta and Ray 2017). Disease causing potential of phytopathogens is often been enhanced due to arthropods feeding (Morrison et al. 1998, Gratwick 1992, Alford et al. 2003, Ray and Hill 2012b) as the feeding scares provide entry point for the fungal pathogens of weeds (Ray and Hill 2016), if not the agents but also by several other fungi which can cause secondary infections. In case of the disease caused by FR where it was instigated with necrosis, but slowly showed signs of chlorosis along the edges of necrotic spots, very indicative of the exposure of the epidermal tissue by the arthropod agents (OT and NB), that are supporting invasion and infection by the pathogen which are inflicting to the death and perish of the weed (Moran 2005, Yamoah et al. 2011). NB and OT may have been useful in

spreading the fungus AA, FO and FR and acting as carriers of mycelial fragments and spores (de Nooij 1988, Kok and Abad 1994), very similar to how *Mycodiplosis coniophaga,* the mycetophagous gall midge, fed on *Puccinia punctiformis* spores and shows significant preference for infected thistles (Kluth et al. 1997). According to previous studies, in most biocontrol programs against invasive weeds, several agents are introduced which would exert a pressure on the invasive species by their additive effects which would be more impactful than on an agent acting singly (Ray and Hill 2016). Such similarity was seen in FR + NB and PS + NB treatments. But, the combination of agents in biological control not necessarily would always lead to better results than the use of a single agent (Hatcher 1995), a lot depends on the species-specific interaction types (Ray and Hill 2016, Dutta and Ray 2017). Similar references where the plant growth was altered in response to the attack of one natural enemy which in turn might impact the performance of the other antagonists, in response to the biochemical changes which can out favour the other agent acting upon the target weed. Hatcher and Paul (2001) also found a negative indirect correlation between herbivory and infection via pathogens, where feeding of the leaf beetle, *Gastrophysa viridula*, on *Rumex obtusifolius* induced a systemic resistance that diminished the overall infection caused by the rust *Uromyces rumicis*. Very similar to the NB and PS clash observed in our experiments, which overall changed the dynamic of waterhyacinth control. Fungal colonization of plants may stimulate changes in the form of morphology, physio-chemistry in host causing modification in the plant quality, altering the insect feeding dynamics (Koricheva et al. 2009). When herbivores and pathogens effect a common host, the overall impact on plant fitness depends on the fractional impact of each of the agents and also on the impact as a result of inter-agent interactions (Dutta and Ray 2017). In the case of (FR + NB)'s and (AA + NB)'s effect on waterhyacinth, after 120 hours, the cumulative effect was less derogative than the damage caused by NB, followed by the fungal pathogen (FR or AA) itself. While OT, on the other hand, facilitated disease development by all the four pathogens. NB's presence slowed down the disease development when treated along with FR and PS respectively. It could have resulted because the weevils has induced the plant defense and/or reduced nutritive value of the host, resulting in retarded impact by the two pathogens or vice-versa (Ray and Hill 2016).

For PS + NB treatment, NB could not establish themselves on PS treated plants resulting in their death, indicating a clear state of repulsion between them, although both individually were antagonistic against waterhyacinth, the targeted macrophyte. This repulsion was further affirmed when the host choice test between the two agents, PS and NB, were performed. The density of the agents plays a pivotal role, where interactions between the biocontrol agents and/on the target plant may not always have a detrimental impact (Denoth et al. 2002, Rayamajhi et al. 2006, April et al. 2011).

Understanding the success of biological weed control, via these interactions is still limited (Hatcher and Paul 2001) and the success is almost not predictable (Crawley 1989). This diverse range of results focuses on further studies on interaction, prior to the release of biocontrol agents on noxious weeds, like waterhyacinth. The multitrophic interactions between the targeted weed and its biocontrol agents provides a much more narrative inference on the inclusive success of a integral weed control programme.

3.5. Conclusion

In the world of increasing development and application of xenobiotic compounds, such as chemical pesticides, using eco-friendly biological control methods become absolute necessity to control aquatic weeds without bioaccumulation or leaving a negative impact on the ecosystem. With the huge array of mycobiota used against waterhyacinth, most have clearly indicated unsuitability as a biocontrol agent from pathogenicity and host-range trials, which could be due to either poor sporulation or low dispersal that requires mostly repeated applying rather than a singular application to ensure a sustainable control (Firehun et al. 2017). However proper insight into the host range test, with proper assessment of safety on non-target plants, along with other potential biocontrol agents, need to be widened before being applied as a potent bioherbicide in the field.