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Molecular characterization and host range studies of indigenous fungus as prospective mycoherbicidal agent of water hyacinth

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ABSTRACT

An indigenous fungal culture, isolated from diseased water hyacinth, in Bolpur, Santiniketan, West Bengal, India, was found to be causing severe blight and dieback disease on water hyacinth, under laboratory and field conditions. It was subjected to morphological and molecular characterization by amplification of 18S RNA gene fragment from genomic DNA using 18S gene universal primers. Subsequently with sequencing, GenBank database comparisons and phylogenetic analysis, the fungus was determined as *Alternaria japonica* Yoshii. Further the pathogen was evaluated for its host specificity to be developed as mycoherbicidal agent against this invasive weed. Host range of *A. japonica* was screened against 48 plant species in 42 genera representing 22 families in pot experiment. Water hyacinth was the only species strongly susceptible to spore suspension (5×10^5 conidia/ml) of *A. japonica*. Minor infection was observed on goosefoot which is not only a weed but also ecologically separated from water hyacinth. Thus, the use of this pathogen in the biological control of water hyacinth would be safe for plants of economic and ecological significance in India. The secondary metabolite produced by *A. japonica* was sprayed on the test plants but phytotoxic symptoms were produced on nine out of 48 plants tested, demonstrating that phytotoxin produced by the fungus is not host specific. Further field tests needs to ascertain its efficacy under more natural conditions.

Key words: *Alternaria japonica*, Biological control, Host specificity, Mycoherbicide, Water hyacinth

Water hyacinth, *(Eichhornia crassipes)*, a native of the tropical South America, is considered to be one of the most serious aquatic weeds (Holms *et al*. 1977). It has spread throughout tropical countries causing widespread problems to millions of users of aquatic bodies and its resources causing severe problems related to its use and management (Gopal 1987). Water hyacinth invasiveness has led to a tremendous negative impact on the social and economic conditions of the aquatic ecosystem, causing a global annual loss of more than US$ 100 million to hydroelectricity generation, irrigation schemes, fisheries, water transport, etc (Shabana 2005).

Various control mechanisms including, manual, mechanical, chemical and biological methods, have been implemented for preventing the invasiveness, or eradication of, water hyacinth by various workers with mixed results (Julien and Orapa 2001, Ray *et al*. 2008). Environmental concerns over the use of chemical herbicides (Ray *et al*. 2008) have drawn interest in biological control of macrophyte. In its native land, water hyacinth is attacked by a large complex of natural enemies including several arthropod agents and fungi (Bennett and Zwölfer 1972, Ray and Hill 2013). But in its range of introduction, in absence of control agents, water hyacinth also flourishes majestically. The biological control agents have provided excellent control of water hyacinth in many locations around the world including India (Center 1994, Coetzee *et al.* 2011).

Biological control of weeds using insects and pathogens has gained considerable importance over last five decades as they are eco- friendly, host specific and effective means of weed control. Among various biological control agents, several phytopathogenic fungi have been found effective against the weed (Charudattan 2001). Various studies have been done to develop these fungi associated with the water hyacinth as potential mycoherbicides (Ray and Hill 2013).

During the present study, a number of indigenous pathogens were isolated from water hyacinth from selected regions of West Bengal (W.B.), India. Among these, a culture of *Alternaria* Nees. (WHK-12), isolated from diseased water hyacinth, in Bolpur, Santiniketan, was observed as a promising mycoherbicidal candidate for biological

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control of water hyacinth during previous studies (unpublished data). Mycoherbicidal potential of several other *Alternaria* species has been reported against water hyacinth from various parts of the world (Nag Raj and Ponnapa 1970, Shabana *et al.* 1997, Pathak and Kannan 2011). *Alternaria* species associated with the weed have also been known to cause severe blight followed by dieback disease to water hyacinth (Nag Raj and Ponnapa 1970, Ray and Hill 2012).

The next step after recovery and screening of potential biocontrol agents is its identification and host specificity test as there is always some risk involved in man's use of new substance or device, be it a drug, pesticide or an electronic device. The isolate was subjected to molecular characterization for identification up to species level. The host range testing schemes have been developed for assessing the safety of non-target plant species against the test pathogen (Wapshere 1974). Thus, an experiment was conducted to determine the host range of this test fungi (WHK-12) by observing the impact of its spore suspension and culture filtrate on 45 plant species in 42 genera representing 22 families.

MATERIALS AND METHODS

Isolation of fungus and culture preparation: The indigenous fungal strain was isolated and purified from the diseased leaves of water hyacinth collected from Bolpur, Santiniketan (Co-ordintes: 23.6700° N, 87.7200° E) in February 2014. The leaves showing disease symptoms were collected and put in large paper envelopes, brought to the laboratory for isolation of pathogens. Back at the laboratory, leaf pieces of about 2 mm^2 was cut from the margins of necrotic or chlorotic lesions on the surface. Pieces were then placed on earlier prepared petri-plates containing potato dextrose agar (PDA) medium and incubated for 3-4 days at 27°C. The fungal species isolated earlier was purified by streak-plate and sub culturing techniques. It was carried out until fresh true monocultures of the fungus were obtained. It was further mass cultured in modified Richard's broth according to Ray (2006) for the present study. The spores were obtained from fungal mat while the toxic filtrate was obtained from the metabolized broth after 21 days of incubation.

Morphological identification of fungus: The fungal strain isolated from infected *E. crassipes*, was morphologically identified by slide culture technique with Lactophenol as mounting medium and observed under Zeiss Axio Scope.A1 Microscope for morphological identification of the genus.

Molecular identification: To confirm the species of the fungi, the isolate was molecularly characterized. Genomic DNA from the fungal isolate mat was extracted by using genomic DNA Isolation Kit (Xcelgen). The DNA stock sample was then quantified using Nanodrop spectrophotometer (Thermo Fisher Scientific NanoDrop™ 1000 spectrophotometer). Purity of DNA was judged on the basis of optical density ratio at 260:280 nm. Concentration of DNA was estimated using the formula.

Concentration of DNA (mg/ml) = OD 260 x 50 x Dilution factor

Agarose 0.8% (w/v) in 0.5X TAE (pH 8.0) buffer was used for submarine gel electrophoresis. Ethidium bromide (1%) was added at 10 µl /100ml. The wells were charged with 5µl of DNA preparations mixed with 1 µl gel loading dye. Electrophoresis was carried out at 80V for 30 min at room temperature. DNA was visualized under UV using UV transilluminator. The DNA concentration and integrity were checked by electrophoresis of the sample on 0.8% agarose gel containing ethidium bromide. After electrophoresis, the agarose gel was photo-documented. 18S RNA gene fragment was amplified from the genomic DNA by PCR (Eppendorf Thermal Cycler), using 18S gene universal primers: 1F and 4R. Details of 18S universal primer sequences were as follows: 1F (CTGGTGCCAGCAGCCGCGGYAA) and 4R (CKRAGGGCATYACWGACCTGTTAT).

Amplified PCR product was then purified using Xcelgen Gel extraction kit, to remove contaminants. To confirm the targeted PCR amplification, 5 µl of PCR product from each tube was mixed with 1 µl of 6X gel loading dye and electrophoresed on 1.2 % agarose gel containing ethidium bromide (1 % solution at 10 ìl/100 ml) at constant 5V/cm for 30 min in 0.5 X TAE buffer. The amplified product was visualized as a single compact band of expected size (Approx 850bp) under UV light and documented by gel documentation system (Biorad). The concentration of the purified DNA was determined and was subjected to automated DNA sequencing BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Sequencing was carried out using BigDye® Terminator v3.1 Cycle sequencing kit. After cycling, the extension products were purified and mixed well in 10 µl of Hi-Di formamide. The contents were mixed on shaker for 30 minutes at 300xg. Eluted PCR products were placed in a sample plate and covered with the septa. Sample plate was heated at 95°C for 5 min, snap chilled and loaded into

autosampler of the instrument. Consensus sequence of 808 bp of 18S region was generated from forward and reverse sequence data using aligner software. The 18S region sequence generated was then used to carry out BLAST with the nr database of NCBI genbank database (http://www.ncbi.nlm.nih.gov; accessed: 17th Dec 2014). Based on maximum identity score, 15 sequences were selected for preparing the phylogenetic tree, constructed using MEGA 5.

The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates (BP) 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 Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 806 positions in the final dataset. Evolutionary analyses were conducted in MEGA5.

Host range studies of test pathogen: The plant species included in the host-range test were selected on the basis of their economic or ecological importance and their relation to the test pathogen or the target plant, water hyacinth. All the test plants taken were at their seedling or early growth stage. They were usually collected from the field during local survey and grown in plastic tubs or cups. All the pots were filled with soil fertilized with farm yard manure and 15-3-12 N:P:K, slow-release fertilizer. The aquatic plants used in the test were grown in similar plastic pots filled with water. For experimentation, plants were kept in the growth chamber conditions at 26 ^{o}C temperature and 75 to 85% relative humidity in plastic pots filled with sterilized soil.

Preparation of spore suspension and phytotoxic culture filtrate: Mass cultivation of the test fungi (WHK-12) was done in modified Richard's broth in ten Erlenmeyer flasks of capacity 1000 ml, each containing 700 ml Richard's broth. These flasks were incubated at 30 ^{o}C in Biological Oxygen Demand (BOD) incubator for 21 days, under condition of 12 hours of alternate day and light. After twenty-one days of incubation, the fungal mat was separated from the liquid metabolized broth, for obtaining spores. The fungal mat was crushed in sterile distilled water and filtered to obtain the spore suspension. Spore suspension (5 x 10^5 spores /ml) was prepared in sterilized distilled water and 0.01% Tween-20 using haemocytometer the metabolized broth was first filtered through eight layers of cheese-cloth then through Whatman No. 1 filter paper to obtain crude culture filtrate to test the phytotoxicity of the secondary metabolite.

Host specificity testing: The test plants were inoculated at the same time during the evening with the spore suspension of the fungi (WHK-12) at concentration 5 x 10^5 spores /ml. They were sprayed until runoff with spore suspension using atomizer. They were covered with transparent polythene bags to create a dew effect for 24 hours and placed in growth racks at 27^{o} C and about 75 to 80% relative humidity. The control plants were sprayed with sterile distilled water and 0.01% tween-20. Another set of similar test plants were sprayed with culture filtrate of the pathogen. All the treatments were replicated thrice. Disease intensity and severity was rated by visual observation at an interval of 24 hours for 30 days. Disease intensity was determined visually on the basis of initiation of disease and increase in disease area seven days after application of the inocula.

RESULTS AND DISCUSSION

Identification and confirmation of species

Morphologically, under microscope, pale brown, simple or branched conidiophores with catenulate conidia at the apex were observed. Section *jponicae* usually contains short to long, simple or occasionally branched primary conidiophores with a single conidiogenous locus (Woudenberg *et al.* 2013). Apical secondary conidiophores were seen to be produced with a single conidiogenous locus. Conidia were porosporous, acropetally developed, dark brown, cylindrical or spindle-shaped, often with cylindrical beaks. Conidia were short, to long-ovoid with transverse and longitudinal septa, conspicuously constricted at most of the transverse septa, in short chains. The species within this section also occur on *Brassicaceae*. Thus the fungal isolate was previously linked to the *A. brassicicola* species-group (Pryor and Gilbertson, 2000, Pryor and Bigelow 2003, Lawrence *et al.* 2013). But this association on being questioned by Hong *et al.* (2005) was later clustered in section *Japonicae* (Woudenberg *et al.* 2013). Further 18S RNA gene fragment was used for characterization and was amplified by PCR from genomic DNA using

18**S** gene universal primers. A single discrete PCR amplicon band of 850 bp was observed when resolved on agarose gel (Fig. 1). Consensus sequence of 808 bp of 18S region was generated (Fig. 2) from forward and reverse sequence data using aligner software. It was then used to carry out BLAST with the nr database of NCBI gene bank database and based on maximum identity score, 15 sequences were selected (Table 1) for preparing the phylogenetic tree, constructed using MEGA 5 (Fig. 3).

The phylogenetic tree is broadly divided into two main clades. The first clade comprises of *Ulocladium botrytis* strain UPSC 3539, *Alternaria cheiranthi* EGS 41-188, *A. alternata* strain HA4087, *A. alternata* strain SRC1lrK2f, *A. maritima* strain CBS 126.60, *A. alternata* isolate AFTOL-ID 1610, *A. alternata* strain FC007, *A. alternata* ATCC 28329, *A. alternata* AA6 and *A. alternata* strain S-f6. While the second clade shows that the closest to our fungal isolate is *A. japonica* strain HDJZZWM- 06 (with 99% identity and 4 BP). Among the same clade the next close ones are *Pleospora herbarum* strain CBS 191.86, *P herbarum*, NS3/NS4 region, *P. herbarum* ATCC 11681 and *P. herbarum* DAOM 150679 respectively. This shows that genetically, *A. japonica* is more closely related to *Pleospora* spp., while among the first clade shows the strains of *A. alternata* and its relatedness with *U. botrytis*. After all these analysis, itt was thus confirmed that the fungal strain isolated, to be similar to *Alternaria japonica* strain HDJZ-ZWM-06 (GenBank Accession Number: GQ354822.1) based on nucleotide homology and phylogenetic analysis.

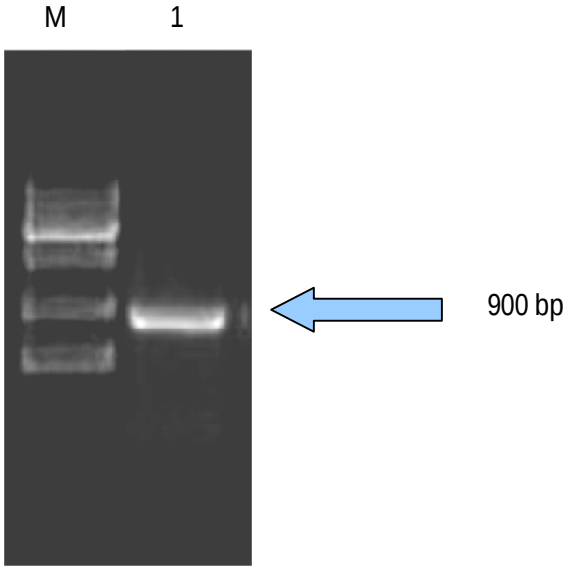

Fig. 1. 1.2% agarose gel image showing single 18S rDNA amplicon of 900 bp after purification by gel extraction. (Lane M: DNA marker (1kb ladder); Lane 1: 18S rDNA amplicon)

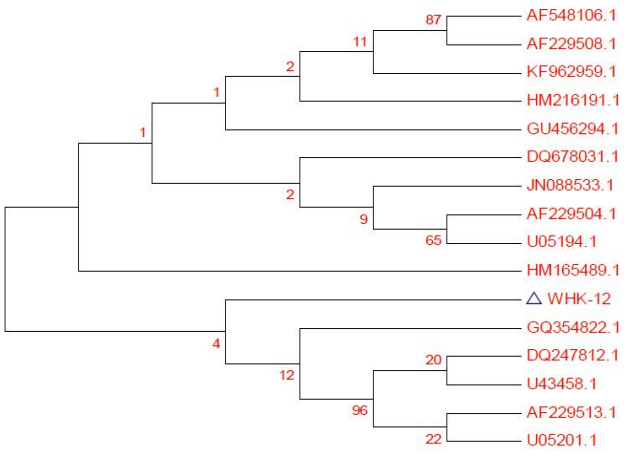

Fig. 3. Phylogenetic tree constructed from 15 closely related sequences, showing similarities between *Alternaria japonica* (WHK-12) and *A. japonica* strain HDJZ-ZWM-06 of Accession Number GQ354822.1. The tree was generated by using the Neighbor-Joining method using MEGA5.

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TAAAGTTGTTGCAGTTAAAAAGCTCGTAGTTGAAACTTGGGCCTGGCTGGCGGGTCCGCCTCACCGCGTGCACTC
GTCCGGCCGGGCCTTCCTTCTGAAGAACCTCATGCCCTTCACTGGGCGTGCTGGGGAATCAGGACTTTTACTTTG
AAAAAATTAGAGTGTTCAAAGCAGGCCTTTGCTCGAATACGTTAGCATGGAATAATAAAATAGGGCGTGCGTTTC
TATTTTGTTGGTTTCTAGAGACGCCGCAATGATTAACAGGAACAGTCGGGGGCATCAGTATTCAGTTGTCAGAGG
TGAAATTCTTGGATTTACTGAAGACTAACTACTGCGAAAGCATTTGCCAAGGATGTTTTCATTAATCAGTGAACG
AAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAACCGTAAACTATGCCGACTAGGGATCGGGCGA
TGTTCTTTTTCTGACTCGCTCGGCACCTTACGAGAAATCAAAGTTTTTGGGTTCTGGGGGGATTATGGTCGCAAG
GCTGAAACTTAAAGAAATTGACGGAAGGTCACCACCAGGCGTGGAGCCTGCGGCTTAATTTGACTCAACACGGGG
AAACTCACCAGGTCCAGATGAAATAAGGATTGACAGATTGAGAGCTCTTTCTTGATTTTTCAGGTGGTGGTGCAT
GGCCGTTCTTAGTTCGTGGGGTGACTTGTCTGCTTAATTGCGATAACGAGCGAGACCTTACTCTGCTAAATAGCC
AGGCTAACTTTGGTTGGTCGCCGGCTTCTTAGAGAGACTATCAACTCAAGTTGATGGA
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Fig. 2. Consensus sequence of *Alternaria japonica* (WHK-12)

Table 1. Fifteen sequences with maximum identity score from BLAST report

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
KF962959.1	*Alternaria alternata* strain HA4087	1493	1493	100%	0.0	99%
JN088533.1	*Alternaria alternata* strain FC007	1493	1493	100%	0.0	99%
HM165489.1	*Alternaria alternata* strain S-f6	1493	1493	100%	0.0	99%
GQ354822.1	*Alternaria japonica* strain HDJZ-ZWM-06	1493	1493	100%	0.0	99%
GU456294.1	*Alternaria maritima* strain CBS 126.60	1489	1489	100%	0.0	99%
DQ678031.1	*Alternaria alternata* isolate AFTOL-ID 1610	1489	1489	100%	0.0	99%
AF229504.1	*Alternaria alternata* ATCC 28329	1487	1487	100%	0.0	99%
U05194.1	*Alternaria alternata* AA6	1487	1487	100%	0.0	99%
HM216191.1	*Alternaria alternata* strain SRC1lrK2f	1483	1483	100%	0.0	99%
AF548106.1	*Ulocladium botrytis* strain UPSC 3539	1482	1482	100%	0.0	99%
DQ247812.1	*Pleospora herbarum* strain CBS 191.86	1476	1476	100%	0.0	99%
AF229513.1	*Pleospora herbarum* ATCC 11681	1476	1476	100%	0.0	99%
AF229508.1	*Alternaria cheiranthi* EGS 41-188	1476	1476	100%	0.0	99%
U43458.1	*Pleospora herbarum* NS3/NS4 region	1476	1476	100%	0.0	99%
U05201.1	*Pleospora herbarum* DAOM 150679	1476	1476	100%	0.0	99%

Table 2. Distance matrix of the 15 sequences with maximum identity score from BLAST report

WHK-12	1		0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.002
KF962959.1	2	0.000		0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.002
JN088533.1	3	0.000	0.000		0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.002
HM165489.1	4	0.000	0.000	0.000		0.000	0.000	0.000	0.001	0.001	0.000	0.002
GQ354822.1	5	0.000	0.000	0.000	0.000		0.000	0.000	0.001	0.001	0.000	0.002
GU456294.1	6	0.000	0.000	0.000	0.000	0.000		0.000	0.001	0.001	0.000	0.002
DQ678031.1	7	0.000	0.000	0.000	0.000	0.000	0.000		0.001	0.001	0.000	0.002
AF229504.1	8	0.001	0.001	0.001	0.001	0.001	0.001	0.001		0.000	0.001	0.002
U05194.1	9	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.000		0.001	0.002
HM216191.1	10	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.001	0.001		0.002
AF548106.1	11	0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.004	0.004	0.002	

Host range of the spore suspension

The present investigation indicates that the indigenous pathogen, A. *japonica* have significantly narrow host range (Table 3). *E. crassipes* and *Chenopodium album* L. (Chenopodiaceae) were the only compatible host plant of *A. japonica* as observed in these studies. None of the other plants were found susceptible to the fungal inoculum. *A. japonica* appears to be a promising biological control agent of water hyacinth. The spore suspension of *A. japonica* also caused appreciable disease on *C. album*. But this does not designate *A. japonica* as a threat as the susceptible plant itself is a noted weed and an ecological difference persists between the aquatic and the land weeds. *A. japonica* has been known to infest Brassicaceae plants including cole crops (Mamgain *et al.* 2013) and cause pod spot of radish (Scott *et al*. 2012). But during the present study none of the plants from Brassicaceae were affected by the pathogen. Thus mycoherbicidal management of water hyacinth seems to have a bright future using the indigenous culture of *A. japonica.*

Host range of the culture filtrate: The culture filtrate of *A. japonica* caused phytotoxic damage to 9 out of 48 plant species tested, *viz*. *Trianthema portulacastrum* L., *Amaranthus viridis* L., *Sinapis alba* L., *Chenopodium album* L., *Spinacia oleracea* L., *Ipomoea aquatic* Forsk, *Hydrilla verticillata* (L. f.) Royle, *Rumex obtusifolia* L., other than *E. crassipes*. Phytotoxic symptoms were produced on several of the plants tested, demonstrating that phytotoxin produced by *A. japonica* is although effective but not host specific. However looking into its damage potential against water hyacinth further intensive studies including proper knowledge of the toxic compounds produced by the fungus is essential. Further using biotechnological approaches (Miller *et al*. 1987) efforts can be directed towards limiting its host range.

Table 3. Host range testing of *A. japonica* on various crops and weed hosts

S.no.	Family	Common name	Vernacular name (in India)	Botanical name	Spore suspension (5×10^5 spores/ml)	Culture filtrate
1.	Aizoaceae	Horse-purslane	Pathar chata	*Trianthema portulacastrum* L.[c]	-	+
2.	Amaranthaceae	Alligator weed	Pani-khutura	*Alternanthera philoxeroides* (Mart.) Griseb. [b c]	-	-
3.		Sessile joyweed	Kantewali santhi	*Alternanthera sessilis* L. [c]	-	-
4.		Amaranth	Chaulai	*Amaranthus viridis* L. [a]	-	+
5.	Apiaceae	Asian pennywort	Brahmi	*Centella asiatica* L. [c]	-	-
6.	Araceae	Water lettuce	-	*Pistia stratiotes* L. [b c]	-	-
7.	Asteraceae	False oxtongue	Kukurbanda	*Blumea lacera* DC [c]	-	-
8.		Chickory	Kasani	*Cichorium intybus* L. [c]	-	-
9.		Parthenium	Gajar ghas	*Parthenium hysterophorus* L. [c]	-	-
10.		Perennial sowthistle	Bhatkataiya	*Sonchus arvensis* L. [c]	-	-
11.		Marigold	Genda	*Tagetes erecta* L. [a]	-	-
12.		Coat buttons	Phulani	*Tridax procumbens* L. [c]	-	-
13.	Brassicaceae[d]	Rai	Sarson	*Brassica campestris* L. *var sarson* [a]	-	-
14.		Radish	Mooli	*Raphanus sativus* L.	-	-
15.		Cauliflower	Phool gobhi	*B. oleracea* L. *var. botrytis* [a]	-	-
16.		Cabbage	Bandha gobhi	*B. oleracea* L. *var. capitata* [a]	-	-
17.		Wild mustard	Safed Rai	*Sinapis alba* L. [c]	-	+
18.	Chenopodiaceae	Goosefoot	Bathua	*Chenopodium album* L. [a c]	+	+
19.		Spinach	Palak	*Spinacia oleracea* L. [a]	-	+
20.	Commelinaceae	Tropical Spiderwort	Kanteri	*Commelina benghalensis* L. [c]	-	-
21.	Convolvulaceae	Bindweed	Hiran chara	*Convolvulus arvensis* L. [c]	-	-
22.		Morning glory	Beshram	*Ipomoea fistulosa* Mart. [c]	-	-
23.		Water spinach	Kalmi sag	*Ipomoea aquatic* Forsk[b c]	-	+
24.	Cyperaceae	Rice foot sedge	Galmotha	*Cyperus iria* L. [c]	-	-
25.	Euphorbiaceae	Asthma weed	Dudhi	*Euphorbia hirta* L. [c]	-	-
26.	Fabaceae					
27.		Gram	Chana	*Cicer arietinum* L. [a]	-	-
28.		Soybean	Soybean	*Glycine max* L. [a]	-	-
29.		Lentil	Masoor	*Lens esculenta* Moench [a]	-	-
30.		Medick	-	*Medicago polymorpha* L. [c]	-	-
31.		Pea	Matar	*Pisum sativum* L. [a]	-	-
32.		Egyptian clove	Barseem	*Trifolium alexandrium* L. [c]	-	-
33.		Mung bean	Moong	*Vigna radiata* L. [a]	-	-
34.	Gramineae	Para grass	-	*Brachiaria mutica* (Forsk.) Stapf. [c]	-	-
35.		Bermuda grass	Dubh	*Cynodon dactylon* L. [c]	-	-
36.		Paddy	Dhan	*Oryza sativa* L. [a]	-	-
37.		Wheat	Gehoon	*Triticum aestivum* L. [a]	-	-
38.		Maize, Corn	Bhutta, Makka	*Zea mays* L. [a]	-	-
39.	Hydrocharitaceae	Hydrilla	-	*Hydrilla verticillata* (L. f.) Royle [b c]	-	+
40.	Lamiaceae	Pignut	Wilayati tulsi	*Hyptis suaveolens* L. Point. [c]	-	-
41.	Lemnaceae	Common duckweed	-	*Lemna minor* L. [b c]	-	-
42.	Linaceae	Linseed	Alsi	*Linum usitatissimum* L. [a]	-	-
43.	Malvaceae	Common wire weed	Kareta	*Sida acuta* Burm. f. [c]	-	-
44.	Polygonaceae	Broad-leaved dock	Jungli palak	*Rumex obtusifolius* L. [c]	-	+
45.	Pontederiaceae	Water hyacinth	Jal kumbhi	*Eichhornia crassipes* (Mart.) Solms	+	+
46.	Solanaceae	Tomato	Tamaatar	*Lycopersicon esculentum* Mill. [a]	-	-
47.	*Verbenaceae*	Wild gooseberry	Pachkotta	*Physalis minima* L. [c]	-	-
		Lantana		*Lantana camara L.* [c]	-	-

[a] Cultivated plant, [b] Plant ecologically related to the test plant, [c] 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

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