Project Report

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Development and formulation of microbial metabolites for the management of root parasitic weed, *Orobanche* spp. in mustard.

Submitted by

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Introduction

Mustard is fourth largest oilseed economy in the world. Among the seven edible oilseeds cultivated in India mustard seed is the second most important oil seed crop in India after soybean. It accounts for nearly 28.6% in the total oilseeds production. The area ofmustard in Madhya Pradesh found to be concentrated in Bhind, Gwalior, Mandla, Mandsaur, Morena andShivpuri districts. These five districts contribute nearly 85 to 90 per cent of area and production of mustard in Madhya Pradesh. Total area under Mustard seed in India for the year 2013-14 is 64.54 lakh with total production of 72.82 lakh tonnes and with an average yield of 1053 kg/hectare during the year. According to the Farmer Welfare & Agriculture Development Department, Madhya Pradesh, the average area of mustard sown was8.14 lakh hectares with the production of 11.60 lakh tonnes and an average yield of 1425kg/hectare during 2014. In India, *Orobanche* spp. has emerged as a major threat to mustard production. Root parasitic weed Broomrape (*Orobanche*) is a major devastating parasitic weed of mustard. Broomrape weed infestation caused 28.2% average reduction in Indian mustard yield. *Orobanche* is a major constraint in the production of mustard, especially in the poorly managed rain fed fields of Western districts bordering Rajasthan which has now spread to the interior central districts of Madhya Pradesh. In addition to mustard, *Orobanche* is found to infect tomato, potato and brinjal, which are the major vegetable crops in the state. Because of their prolonged underground survival and parasitism, attempts to manage the weed by chemical or other methods have not been successful till date. Further their intrinsic relationship with the host has prevented development of any successful chemicals against this dreaded weed. *Orobanche* enjoys a very intimate relationship with the hosts and thus any management strategy of these parasites should be aimed at interfering through their physiological processes of their interactions like signal disruption, or release of active oxygen species in the hosts by inducing their resistance. So far there are no resistant varieties available against the parasitic weed. Further by the time the weed is identified by the above ground, much damage has already been done to the crop. Use of secondary metabolites from native soil fungi will help in the biological management of the weed. Thus it is expected that the metabolites from the fungi would also have a detrimental effect on the infection process of the parasitic weeds. This strategy is expected to involve the following modes of action:

- Germination stimulant for suicidal germination and resulting reduction in the weed seed bank for the succeeding crop.
- False signaling by the host to the parasite movement to avoid its contact

- Direct suppression of the parasitic process attachment of the haustoria and its subsequent invasion of the host
- Induce systemic resistance in the host.

In this context the use of secondary metabolites from native rhizospheric microbes to directly suppress the infection processes of the parasitic weeds and induce systemic resistance in the host plants, have been thought of as a better management strategy. The project proposes the management of the dreaded root parasitic weed *Orobanche* in mustard as an environment safe and can be applied easily at low doses. Thus the project aims at development of a partially purified potential microbial secondary metabolite that is suitably formulated for their use by the farmers against *Orobanche* in an ecologically safe manner. Thus the proposed project was started with the aim to manage the root parasitic weeds at three stages viz., (i) seed stage (ii) at infection stage and (iii) at the establishment stage.

Objectives

The objectives of project are given below:

1. Extraction of crude fractions of secondary metabolites from the commonly occurring soil fungi e.g., *Trichoderma* spp., *Fusarium* spp., antagonistic against *Orobanche* spp.
2. Bioassay screening of isolated potential fungi and their crude fractions on *Orobanche* and to analyze the effect of systemic resistance elicited in the host plants.
3. Proteomics of the interactions of the host plant, parasitic weed and the microbial metabolite for better understanding of the resistance process of the host
4. Partial purification and formulation of the potential crude fraction for their sustained and safe release in the farmer's field.

Annual work plan

The work completed during the period of project is given below in the form of annual work plan.

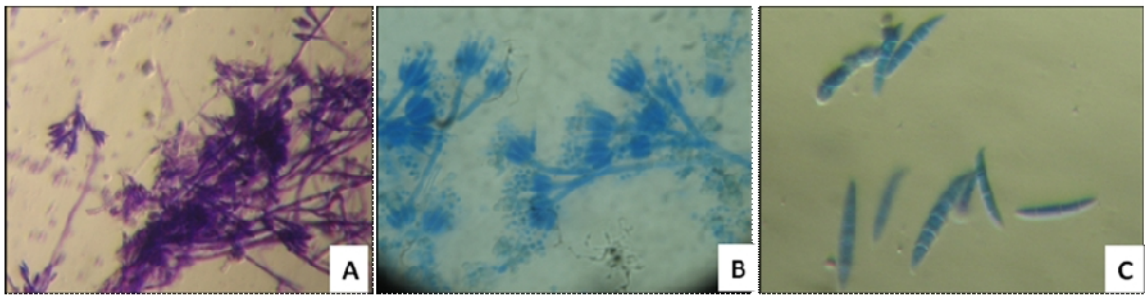
Item of work	I Year			II Year			III Year		
	I	II	III	I	II	III	I	II	III
Isolation of microbes from the farmers field infested with *Orobanche*	√	√	√						
Extraction of cell free culture extracts from the microbes		√	√	√					
Bio-efficiency screening (germination tests, analysis of infection processes and estimation of induced systemic resistance)				√	√	√			
Partial purification of the crude fractions							√	√	
Formulation of the purified metabolite								√	√
Analysis and report writing									√

Work done

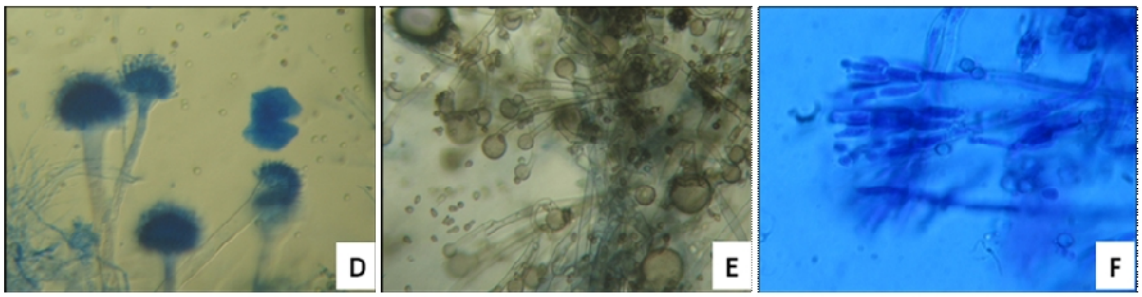
According to the annual work plan, the following work has been completed during the period of project from March 2012 to March 2015. The work done report with its achievements is given under the individual objective in the following sections

I. Isolation of native antagonistic fungi from the farmers field, infested with *Orobanche:*

- Soil samples were collected from *Orobanche* infested soils of western MP (Gwalior and its nearby districts *viz.,* Bhind, Murena) and Bharatpur, Rajasthan during 2012. The samples were screened for microbial populations.
- The microbes were isolated by serial dilution technique and pure cultures were obtained by hyphal tip techniques from the mixed cultures in the first step and then maintained in PDA media under refrigerated conditions for further use.
- *Trichoderma, Gliocladium, Fusarium* sp., *Pythium* sp., *Penicillium* sp., and *Aspergillus* sp., were identified by their morphological characters. Microbes were stored in PDA slant at 4^0C. **(Fig 1)**.



A. *Trichoderma viride*, B. *Gliocladium virens*, C. *Fusarium* sp



D. *Aspergillus* sp., E. *Pythium* sp. F. *Penicillium oxalicum*

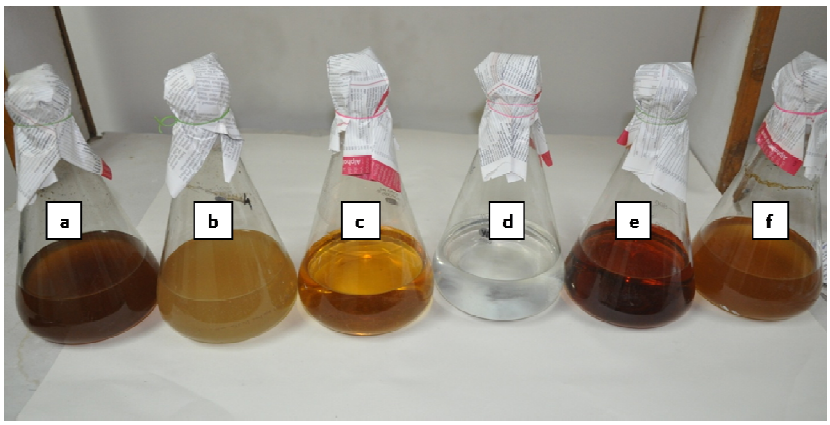
Fig. 1. Microbes isolated from rhizosphere soil of *Orobanche* infested mustard.

- Microbes were stored in PDA slant at 4^0C (Fig. 2.).

Fig.2. Different culture isolated from different fields infested with *Orobanche*

II. Standardization of media for the better crude fraction extraction of cell free culture extracts from the microbes:

- Different broth media were prepared for the fungal growth. Following media were tried for growth and extraction of cell free culture (Fig.3.).
    a) Richard's Broth
    b) Malt extract broth
    c) Potato dextrose broth
    d) Potato sucrose broth
    e) Dextrose peptone broth
    f) Dextrose broth.



a. Potato sucrose broth**, b.** Dextrose broth**, c.** Dextrose peptone broth**, d.** Richard's Broth**, e.** Malt extract broth**, f.** Potato dextrose broth**.**

Fig.3. Different culture broth for standardization of better production of crude fraction of metabolites.

- Individual fungi were cultivated with different culture media to find out the best medium for cultivation of the fungi to produce better quantity and quality of the compounds.
- Isolated microbes were inoculated in different broths as mentioned above in 1000 ml conical flasks and kept at 28±2^{0}C for 2 weeks.
- After standardization of the culture media, all the microbes were grown in 1000ml Potato dextrose broth and further incubated for 21 Days for the proper growth.
- Crude fraction of the Secondary metabolite from the microorganisms were isolated using different solvents on the cell free culture filtrate of 21 days old microbial culture in potato dextrose broth medium. The crude was used for bio-efficacy studies and was stored under -20ºC for further purification
- Extraction of crude fraction **(Fig.4.)** was standardized by using different solvent viz, Dichloromethane, Ethyl acetate, Chloroform and Acetone and it was found that Dichloromethane is quite good when compared with other solvents and hence used for the crude fraction extraction.
- The extracted crude fraction was weighed to find out the yielding potential of different microbes. Accordingly the extracts were dried under room conditions and weighed in precision balance.

Fig.4. Solvent Extraction set up for microbial cell free culture extraction.

- The following **Table 1**gives the yield of the crude extracts from microbial cultures obtained by solvent extraction using DCM.

Table 1. Crude extracts of the antagonistic fungi isolated from the rhizosphere soils of *Orobanche* infested mustard.		
Microbe	**Broth Used (ml)**	**Amount of Crude extract**
Fusarium sp.DWSR2 Gwalior	1000	1.494 mg
Fusarium oxysporum DWSR1	1000	1.154 mg
Penicillium oxalicum DWSR1	1000	3.605 mg
Pythium sp.	1000	1.544 mg
Trichoderma viride	1000	7.236 mg
Gliocladium virens	1000	0.922 mg

III. Bioassay screening of the crude fractions for their direct action on *Orobanche* and to elicit systemic resistance in mustard.

a) Development of germination setup for *Orobanche* seeds under *in vitro* conditions

Orobanche seeds from fully matured flowers of *Orobanche* growing on mustard crops were collected from the farmer's field in Gwalior district Madhya Pradesh, India, during March 2012. The seeds were sun dried and stored in plastic containers at room temperature (25±2ºC).Seed viability was assayed using 2, 3.5-triphenyl-tetrazolium chloride (TTC) staining (Granados and Torres 1999), for which the seeds were placed in 1% solution of TTC and incubated for 72hr at 35ºC in the dark. The incubated seeds were then observed microscopically, red and orange color seeds were considered viable while white color seeds were considered as non viable seeds or dead seeds. Surface sterilized seeds, about 100 in numbers were placed on autoclaved moist filter paper of size Whatman No.1 in Petriplates of diameter 9 cm. The plates were covered with black polythene sheets and wrapped in aluminum foil to provide complete darkness and then incubated at 25±2 ^{0}C for 10 days for conditioning (Plakhine *et al*., 2009). The preconditioned seeds with filter paper were aseptically transferred on a bed made of sterilized sand, cotton and filter paper kept on a 1000 ml beaker. In a parallel setup, surface sterilized seeds of mustard were placed in separate Petriplate with moistened blotting papers for germination. After germination, about 10 seedlings was surface sterilized and transferred to the beaker containing the preconditioned *Orobanche* seeds. A mild fungicide such as bavistin was sprayed to avoid fungal contamination. *Orobanche* seeds without host plant were maintained for the purpose

of comparison. About 30 beakers with similar sets of *Orobanche* seeds and host plant seedlings were maintained (Aditi and Kannan 2014)

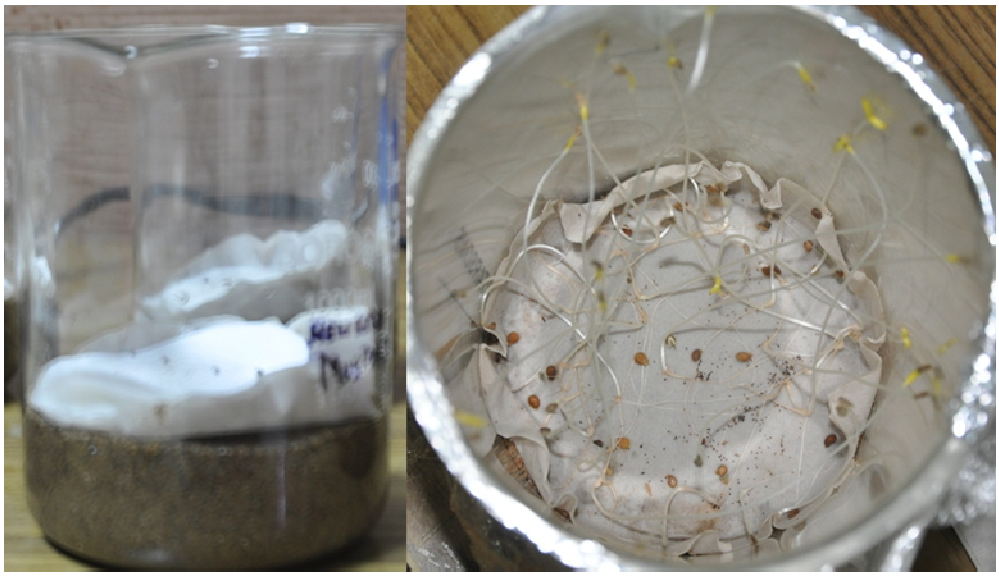


Fig 5. Setup for germination of *Orobanche cernua* under controlled conditions

The beakers were then incubated at room temperature for 10 days after which, the filter paper with *Orobanche* seeds and host plant was removed gently for observation under stereo binocular microscope.

Microscopic observation of the germinating seeds indicated the following important events in that sequence as follows:

i) Seeds swell after absorbing moisture during the conditioning period
ii) Color of the seed coat changes from light brown to dark brown on 2nd to 4th day upon exposure to the host seedlings.
iii) The proximal end of the imbibed seeds became more pointed and protruded from the base
iv) The testa ruptured
v) Germ-tube elongates to reach the host root and attaches to the root surface by production of haustoria to enter the root tissues.

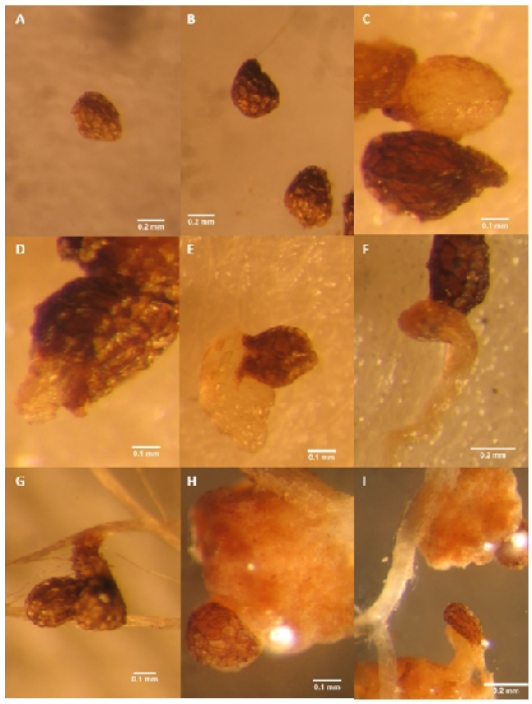


Fig. 6. Micro-photograph showing different stages of germination of *O. cernua*
A. Normal seed with reticulation B. Swollen and conditioned seeds, C. Imbibed with moisture and initiating germination at the proximal end of the seed, D. Bursting of seed coat (testa) from the proximal end of the seed and formation of germ tube, E. Development of germ tube, F. Germ tube elongation for host root search, G. Germ tube attaching to the host root and establishment of parasite to the host root, H. Tubercle development, I. Two stages of establishment of parasite, germ tube and tubercle development.

After successful establishment of haustoria inside the host root, a globular tubercle is formed. The tubercle is densely surrounded by brown root meristem, growing into a flowering stalk of *Orobanche* which emerges above the ground to produce flowers.

Table 2. Viability of *Orobanche* seeds collected from the soils of different villages around Gwalior and tested by TTC staining method

Sample	**Host crop**	**% Viability of *Orobanche* seeds**
Bahadurpur 1	Mustard	65.8
Khokapura 1	Mustard	49.2
Murena 2	Mustard	67.8
Bahadurpur 2	Mustard	56.6

Source: Aditi and Kannan 2014

The germinated host plant seedling provides the essentially required host signals in a concentrated manner right near the pre-imbibed *Orobanche* seeds, thereby providing a perfect condition for the germination and growth of *Orobanche* seeds.

Table 3. Comparison of some popular methods for weed seed bank estimation

Objective of the technique	**Advantages**	**Disadvantages**	**Reference**
In vitro germination of parasitic weed seeds	Can provide valid information about the seed bank load of the parasitic weeds	Root leachate collection and tissue culture involves costly facilities, expertise and time	Batchvarova *et al*. (1998)
Sandwich model of *Orobanche* germination	Can provide a general information about presence of *Orobanche* seeds	Does not give complete information, time consuming and the glass-fiber may cause respiratory problems for the personnel involved	Losner-Goshen *et al*. (1998)
Petridish method using synthetic germination stimulants like GR24, Nijmegen-1 etc.,	Widely used technique for the study of *Orobanche* seed germination Gives better germination than any other technique under controlled conditions	Germination stimulants are costly and not readily available in the market No information about complete life cycle, only germ tube development can be observed but tubercle development and attachment will not happen because of absence of host root	Mangus *et al*. (1992)
Seed germination by using live host seedlings under laboratory conditions Quantification of soil seed bank Screening of crop	Germination of the parasitic weed seed, interaction between the host plant and weed and stages of development of the parasitic weeds can be studied in relatively less time Uses locally available standard laboratory wares and thus very	Host plant may become week because of lack of food sources Takes about 20-25 days including the preconditioning and germination period in the case of soil seed	Our proposed technique

seed samples from infested fields	cheap and easy to setup when compared with other methods Can be used to quantify the soil seed bank load of parasitic weed seeds which is not possible by other methods Depending upon the sample size and representativeness, can give accurate prediction of the seed load in the soil Screening of crop seed lots is less laborious and reliable than other methods	bank screening.	

Source: Aditi and Kannan 2014

b) Biology of *Orobanche cernua*

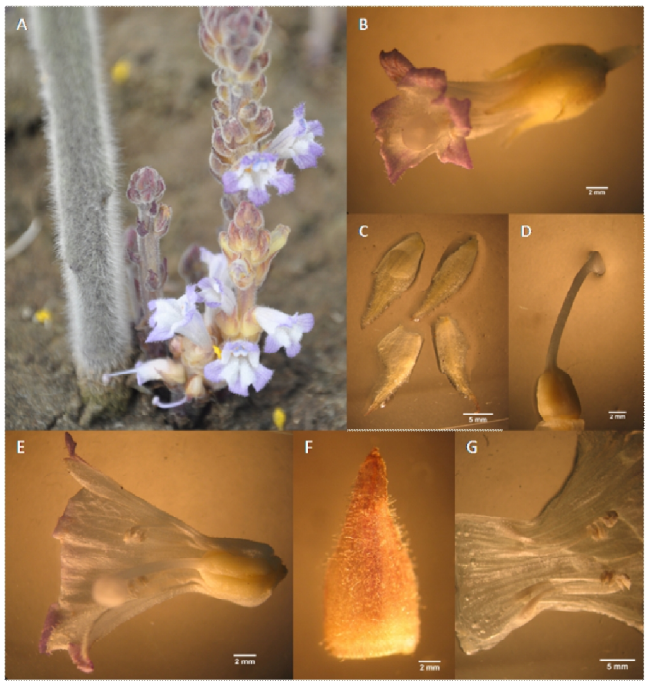


Fig.7. Micro-photographs showing key characters of *O. cernua* :
A. Stalk with flowers, B. Single flower, C. Calyx segments, D. Pistil, E. Corolla with stamens and ovary, F. Bract, G. Open corolla with four stamens.

O. cernua is commonly known as nodding broomrape has leafless flowering stems 15-40 cm high bearing alternate scales less than 1 cm long. Although usually unbranched above ground, multiple stems sometimes arise from a single tubercle below ground. The plant is pale, completely lacking any chlorophyll. The base of the stem, below ground, is normally swollen and tuberous. The inflorescence, occupying up to half the length of the stems carries many acropetally developing flowers, arranged in spikes or racemes, each

subtended by a bract 7-12 mm long. The calyx has four free segments, more-or-less bidentate, 7-12 mm long. The white corolla tube, 12-30 mm long, is inflated near the base, conspicuously down-curved, with narrow reflexed tips, up to 10 mm across. The tube is mainly white or pale while the tips are contrastingly blue or purple, without distinct venation. Filaments are inserted in the corolla tube, 4-6 mm above the base. A capsule develops up to 8-10 mm long and may contain several hundred seeds, each about 0.2 x 0.4 mm.

It is an obligate parasite which establishes its connection with the host root within a few days of germination. The seed of *Orobanche cernua* is minute (approximately 0.2 x 0.4 mm), from which only the radicle emerges growing only a few mm long. A chemical stimulus which is exudated by the host roots is needed to trigger *Orobanche* germination. Therefore, *Orobanche* normally germinates only when a host root is nearby. However, a moist environment is required (for several days) together with suitable temperatures, before the mature seed is responsive to germination stimulants. This preparatory period is known as conditioning or preconditioning. Conditioned seeds remain responsive to germination stimulants for a limited period beyond which secondary dormancy may be induced, especially at lower than optimal temperatures (Weldeghiorghis and Murdoch, 1997; Kebreab and Murdoch, 1999a). Their ability to respond to germination stimuli also fades gradually when the seeds dry and they then remain dormant until reconditioned (Timko *et al*., 1989; Joel *et al*., 1995). On contact with the host root, a swelling, the haustorium, is formed, and intrusive cells penetrate through the cortex to the vascular bundle to establish connection with the host xylem. This process is assisted by the exudation of pectolytic enzymes. The parasite develops into a tubercle on the surface of the root, developing to a diameter of 5-20 mm. Secondary roots may develop on the tubercle and make separate contacts with the host root system. After several weeks, the tubercle develops a flowering shoot which emerges above the soil. Seeds are produced in very large numbers and remain viable in soil for many years, in many situations. *Orobanche* spp. depend totally on their hosts for all nutrition, drawing sugars and nitrogen compounds from the phloem and also drawing most of their water from the host xylem. The parasite becomes an active sink, comparable to an actively growing part of the host plant itself, such that effects on the host are generally proportional to the biomass of the parasite.

c) Trials for standardizing the conditions for germination of *Orobanche* in pots:

- *Orobanche* seeds were collected and kept for conditioning in soil for 2 months. Conditioning was done by mixing the seeds in soil and daily irrigation.
- After 2 months mustard seeds were sown in the same pots.
- *Orobanche* emergence was recorded after 40- 50 days of sowing of mustard **(Fig. 8).**

Fig.8. Trial for *Orobanche* emergence in containment (Pot experiment)

d) Bio-efficacy studies of metabolite fractions

- Bioefficacy study was performed to analyze the effect of extracted crude extracts of the biocontrol fungi on germination of *Orobanche* seeds, infection processes and estimation of induced systemic resistance in the host plants.
- For studying the bio-efficacy of the metabolites microbial culture preparation was prepared for the extraction and mass multiplication of secondary metabolites.
- Microbial cultures were grown in pure cultures in 4 L culture flasks for 21 days and then extracted using the method standardized at DWSR, using Dichloromethane solvent **(Fig.9.)**.

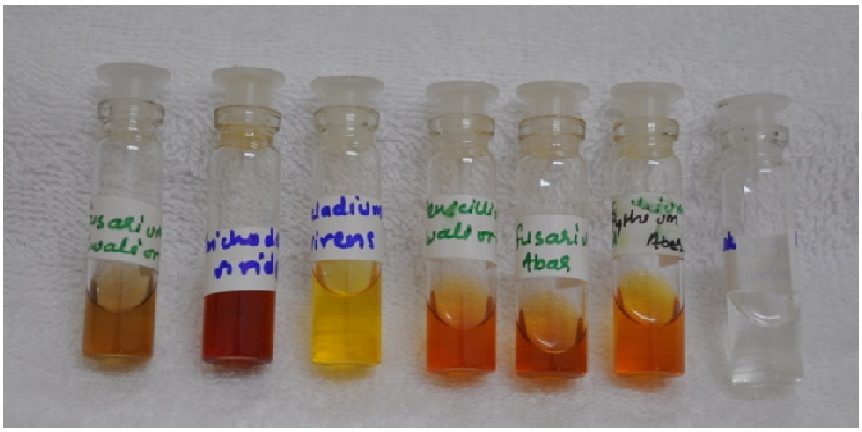


Fig.9. Crude Extracts isolated from different microbes.

- The metabolites were run on TLC to confirm the presence of active metabolite compounds.

Columns from Left to right

1. *Fusarium oxysporum* DWSR1 Isolated from Abar.
2. *Fusarium* sp Isolated from Gwalior
3. *Penicillium oxalicum* DWSR1
4. *Pythium* sp
5. *Trichoderma viride*
6. *Gliocladium Virens*.

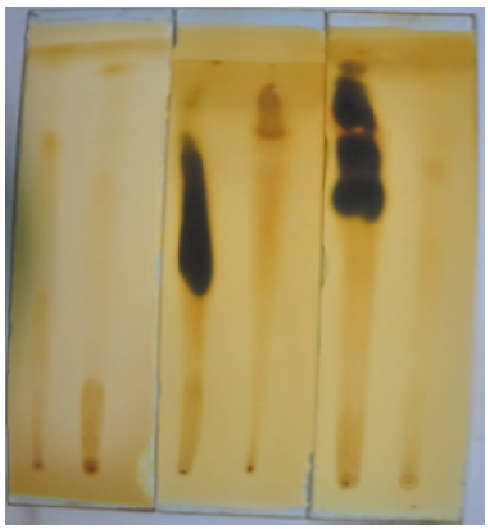


Fig.10. TLC of Extracts from Isolated microbes cultured on different broth media

- The metabolites were applied to the seeds of *Orobanche* to analyze its capability to suppress germination of *Orobanche* seeds under laboratory conditions).
- The potential compounds were analyzed using LCMSMS to further characterize them.
- Bio-efficacy studies were performed using a simple and rapid technique to germinate *Orobanche* under laboratory conditions as per standard procedure developed by DWSR.
- Host Seeds were treated with microbial metabolites and seedlings were transplanted to the beakers containing preconditioned *Orobanche* seeds on Whatmann filter paper grade 1.
- Observations were made for number of *Orobanche* seeds germination.
- Results (Table 4) explain that among all isolated microbes *Fusarium oxysporum* DWSR1, *Fusarium* sp. DWSR2 and *Penicillium oxalicum*DWSR1were able to inhibit germination of *Orobanche* seeds when treated with crude extracts.

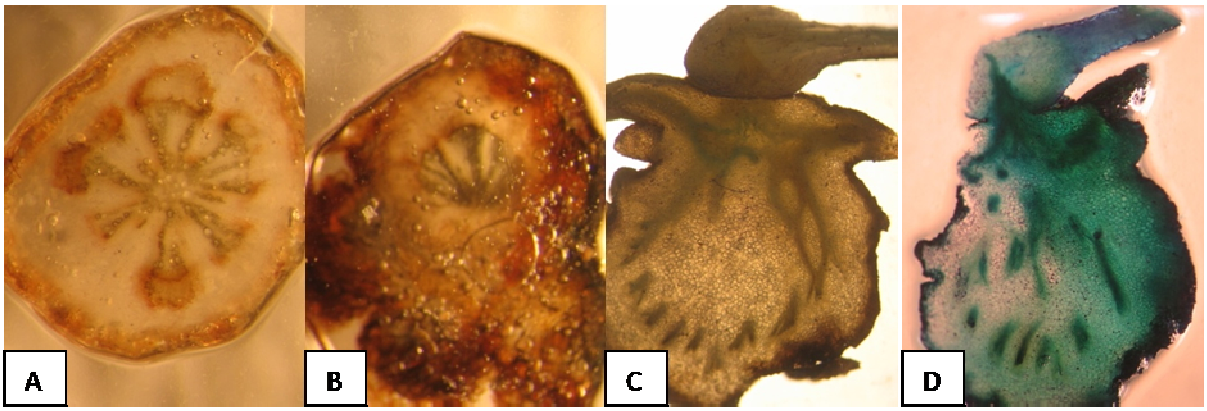
Table 4: Effect of crude metabolite fraction on germination of *Orobanche*

Treatments	% germination of *Orobanche* in % concentration of crude extracts			
	5%	10%	20%	50%
*Fusariumoxysporum*DWSR1	19.67	17.33	13.33	11.67
Fusarium sp. DWSR2 (Gwalior)	21.33	19.33	17.33	15.67
Penicillium oxalicum DWSR1	23.33	20.33	16.67	14.67
Gliocladium virens	32.67	30.67	29.67	28.67
Trichoderma viride	27.67	25.67	24.67	23.67
Control	46.67	51.67	53.67	54.67

e) Infection processes as studied under microscope:

- The lifecycle of *Orobanche* is highly specialized for parasitic lifestyle, comprising a non-parasitic phase and the parasitic phase.
- The first phase consists of germination of the seed as a response to the host signals, followed by a short duration of germ tube development towards the host root, formation of the haustorium and the appresorium, penetration of the host tissues and connection to the vascular tissues.
- During the second phase, the parasite grows at the expenses of the host, developing a small tubercle at the beginning, a crown of roots and a flowering stalk, which emerges above the soil to flower and set seed.
- *Orobanche* tubercles attached to the tomato root were processed by thin hand sections and were fixed in FAA and were dehydrated through a series of ethanol concentrations.
- The sections were treated with Toluidine blue in phosphate buffer. Observations says that after penetrating the host root the haustoria forms a nutrient bridge for the supply of nutrients from host to parasite.
- It was also observed that *Orobanche* produces primary haustoria which enter the root tissues and secondary haustoria consisting search hyphae behind the primary, for the search of adjacent host vascular tissues.

- The host cortex tissues upon attack by the haustoria turned brown indicating an increased accumulation of the phenolics as a result of the host reaction.



A. Healthy root of mustard, B. Infected root of mustard, C. C.S. of *Orobanche* tubercle. D. C.S. of *Orobanche* attached wit host vascular bundle

Fig.11. Microscopic view of *Orobanche* infection on mustard

f) *Orobanche* infestation on mustard subjected to various treatments

- The experiment was conducted in pot and was studied under containment facility.
- The details of the treatment given to mustard are given below in **Table 5**:

Table 5. Treatment details		
T1	*Fusariumoxysporium*DWSR1	Seed treatment and foliar spray on 30-60 DAS
T2	*Penicilliumoxalicum*DWSR1	Seed treatment and foliar spray on 30-60 DAS
T3	*Fusarium* spp. gwalior	Seed treatment and foliar spray on 30-60 DAS
T4	*Fusariumo xysporium* DWSR1 metabolite	Root drenching on 30-60 DAS
T5	*Fusarium spp.* Gwalior metabolite	Root drenching on 30-60 DAS
T6	*Penicillium oxalicum* DWSR1 metabolite	Root drenching on 30-60 DAS
T7	Negative Control	Host + *Orobanche*
T8	Positive Control	Only Host

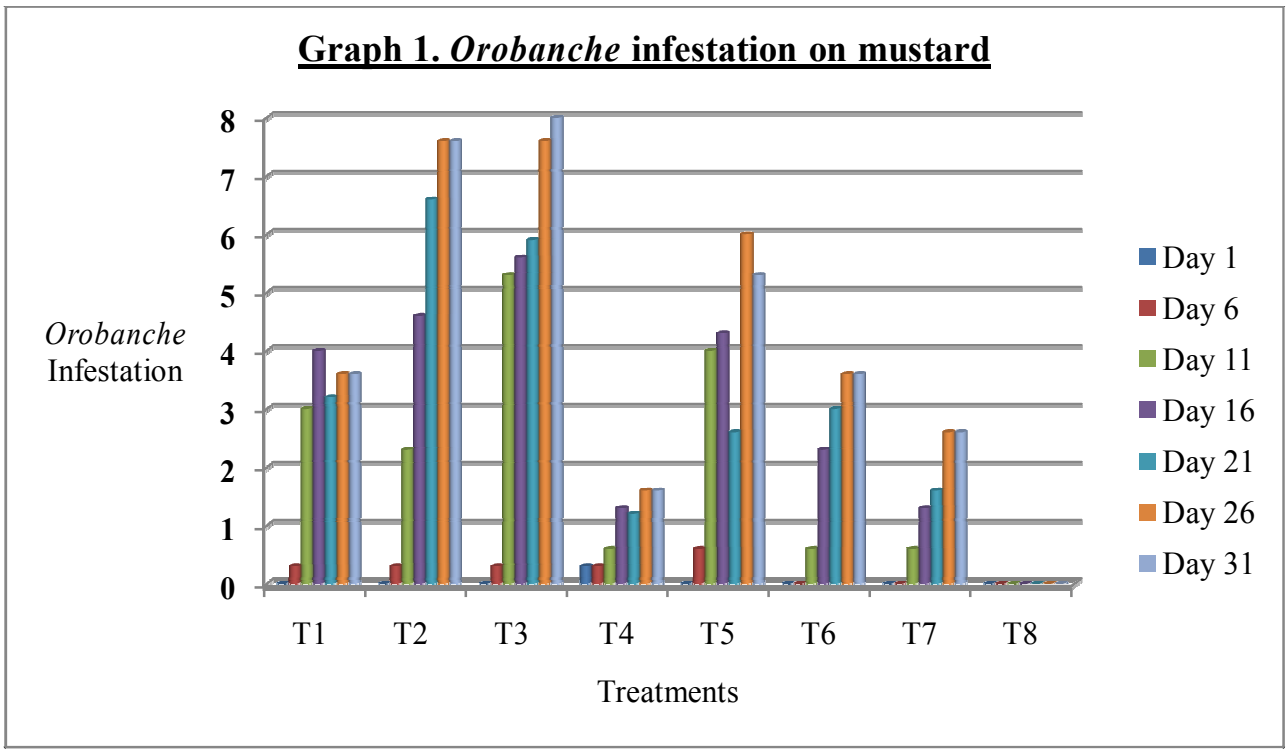
- Number of *Orobanche* infested on mustard at an interval of 5days are recorded in the form of **Table 6**.

Table 6. Number of *Orobanche* infestation on mustard for various treatments

Treatment	*Orobanche* infestation						
	Day 1	Day 6	Day 11	Day 16	Day 21	Day 26	Day 31
T1	0	0.3	3	4	3.2	3.6	3.6
T2	0	0.3	2.3	4.6	6.6	7.6	7.6

T3	0	0.3	5.3	5.6	5.9	7.6	8
T4	0.3	0.3	0.6	1.3	1.2	1.6	1.6
T5	0	0.6	4	4.3	2.6	6	5.3
T6	0	0	0.6	2.3	3	3.6	3.6
T7	0	0	0.6	1.3	1.6	2.6	2.6
T8	0	0	0	0	0	0	0

- On the basis of the data collected, a graph was plotted for seeing the effect of various treatment on *Orobanche* and result was interrupted.



- According to the graph

1) *Fusarium oxysporium* DWSR1(T1) treatment has resulted in less *Orobanche* infestation as compare to other two bioagents.
2) *Fusarium oxysporium*DWSR1metabolite (T4) and *Penicillium oxalicum*DWSR1 metabolite (T6) delayed *Orobanche* infestation in comparison to *Fusarium* Gwalior DWSR2.

g) Molecular identification of the potential two fungi

Apart from identifying the fungi based on the morphological characters, the following procedure was followed to identify the DNA pattern to confirm the identity using the conserved pattern of 18SrDNA.

1. DNA was isolated from the culture provided by the scientist. Its quality was evaluated on 1.2% Agarose Gel, a single band of high-molecular weight DNA has been observed.

2. Fragment of 18S rDNA gene was amplified by PCR from the above isolated DNA. A single discrete PCR amplicon band of 900 bp was observed when resolved on Agarose Gel (Gel Image-1).
3. The PCR amplicon was purified to remove contaminants.
4. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 1F and 4R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer.
5. Consensus sequence of **850bp** 18S rDNA gene was generated from forward and reverse sequence data using aligner software.
6. The 18S rDNA gene sequence was used to carry out BLAST with the database of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 5.

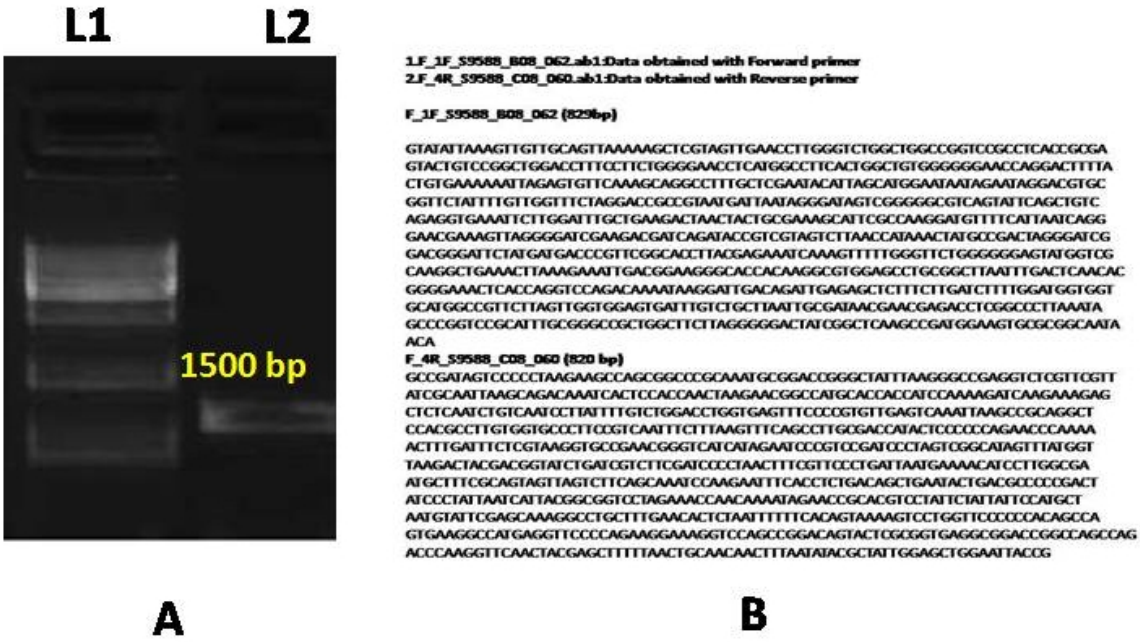


Fig.12. A. L1.Marker protein, L2.18S rDNA amplicon.
B. Nucleic acid sequence of *Penicillium oxalicum* DWSR1

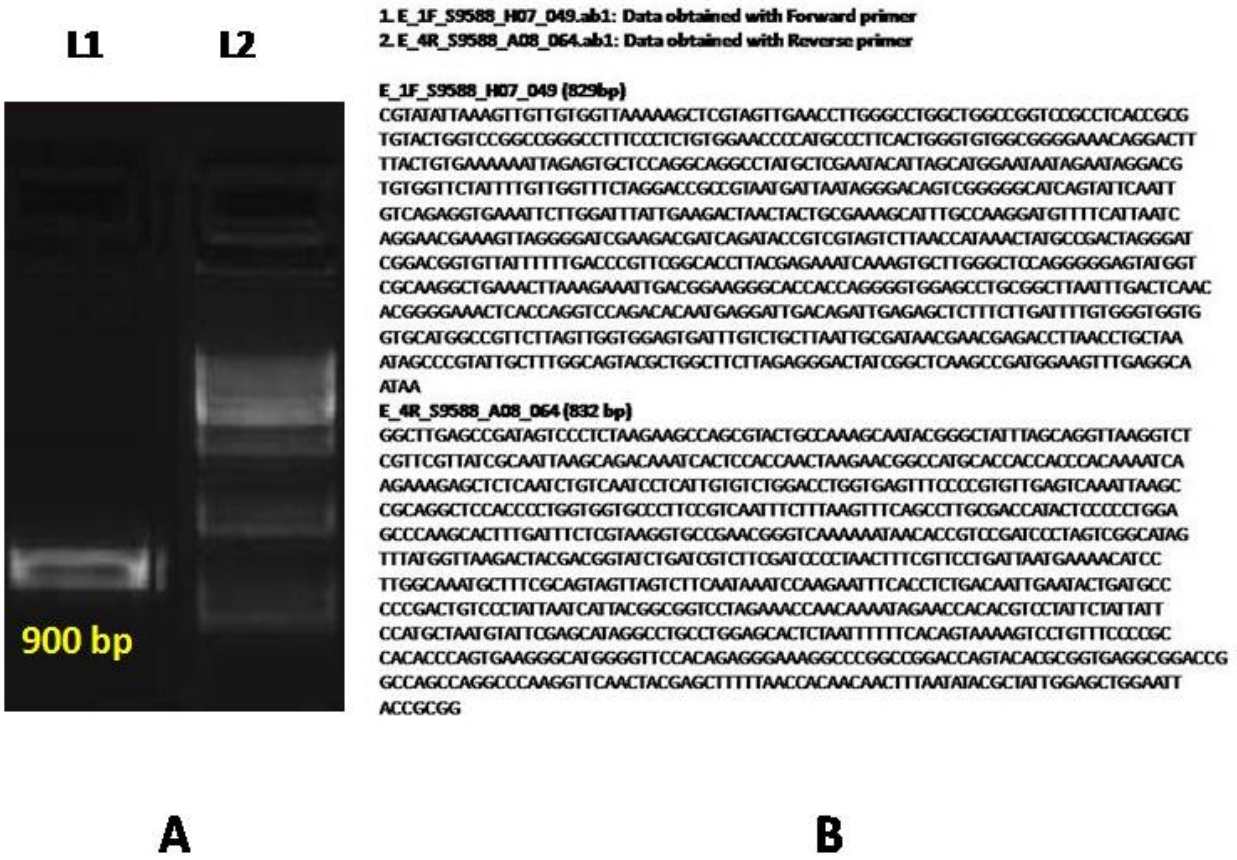


Fig.13. A. L1. Marker protein, L2.18S rDNA amplicon.
B. Nucleic acid sequence of *Fusariumoxysporum*DWSR1

h)Analysis of induced systemic resistance in host plants

Systemic resistance in host plant was quantified by the estimation of the enhanced activities of the defence enzymes upon application of the bio agents and the crude fraction of their metabolite. Defence enzymes involved in host resistance when infected by the parasites viz., peroxidase, polyphenol oxidase, catalase and phenylalanine ammonia lyase was analyzed using spectrophotometer. These enzymes are inherently produced in the plants, but their activities are enhanced when the applied fungal pathogens act as elicitors to induce their production. The enzyme values of mustard after 30 days of sowing (DAS) with *F.oxysporium*DWSR1and *P.oxalicum*DWSR1 1is given in **Table 7**.

Table 7.Defense enzymes activity estimated from mustard after elicitation by the bioagents.

Parameters	Peroxidase Activity (ΔOD/mg of protein/min)	Phenylalanine ammonia lyase Activity (µmol of trans-cinnamic acid/mg protein/h)	Polyphenol Oxidase Activity (ΔOD/mg of protein/min)	Catalase Activity (µ mol of H_2O_2/ min/ mg of protein)
*Fusariumoxysporium*DWSR1	10.10	7.04	4.75	2.60
Penicilliumoxalicum DWSR1	9.95	7.10	4.45	2.70
Control	10.20	7.50	4.70	2.75
CD @ 5%	0.82	0.65	0.45	0.30

Based on above results it was concluded that the fungal bioagents were not able to elicit significantly high defense enzymes *viz.,* peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and catalase more than the control plants. Hence these were not subjected to further systemic induced resistance studies.

IV. Proteomics of the interactions of the host plant, parasitic weed and the microbial metabolite for better understanding of the resistance process of the host

As a part of understanding the infection processes, the proteins involved in the infections process and the proteins either suppressed or activated during the process, a study was planned to collect the tissues from the point of attachment of the host and the parasite and send for analysis. Accordingly the proteins were extracted as per standard protocol, run in the PAGE and the band portion showing maximum density was eluted and sent for analysis to the Xcelris (I) limited. However it was learnt that the eluted bands did not have proper protein or that the proteins were degraded. Hence currently this objective could not be completed.

Partial purification of the crude fractions:

The pure lines of *Fusarium* collected from mustard fields of different places were grown on potato dextrose broth for 30 days. The broth was then extracted in ethyl acetate and concentrated under vacuum. The concentrated *Fusarium* extract was analyzed by LC-MS/MS. Similarly, the media of *P.oxalicum* DWSR1 culture was also extracted, cleaned up

and analyzed by LC-MS/MS. From the total ion chromatography the tentative molecular weight of major metabolites were assigned and given below:

- Extract of *Fusarium* spp DWSR2 : 447
- Extract of *Fusarium oxysporum* DWSR1 : 165, 278, 317, 645
- Extract of *Penicillium oxalicum* DWSR1: 566, 610, 654.

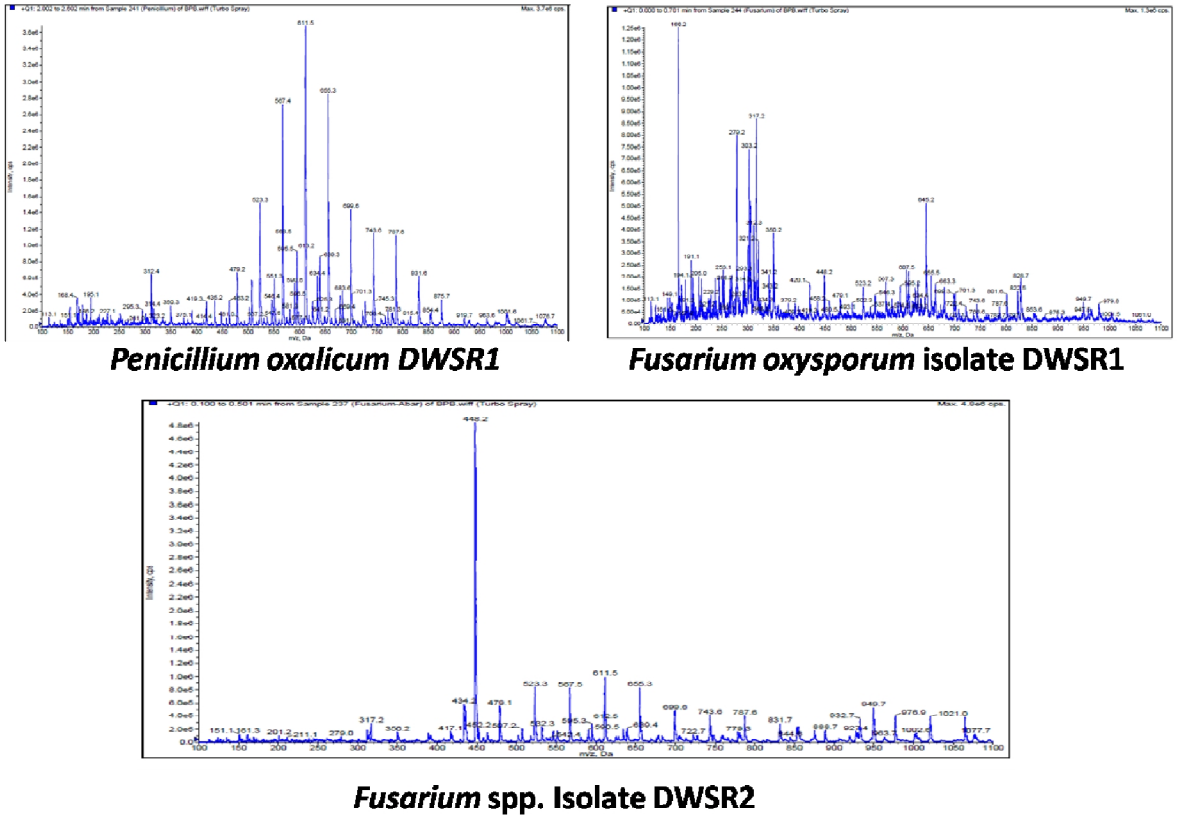


Fig.14. Total ion chromatogram of different microbial extracts.

Formulation of the crude fractions

Materials: The chemicals and solvents were procured from E. Merck India Ltd. The crude extracts from *Penicillium oxalicum* DWSR1and *Fusarium oxysporum* DWSR1 were obtained by solvent extraction of media where the fungi were allowed to grow separately.

Preparation of EC formulations: Formulations with six different combinations **(Table 8)** of solvents and adjuvants were tried in formulating the bioactive crude microbial metabolites obtained separately from *Penicillium oxalicum* DWSR1 and *F.oxysporium* DWSR1. The crude extracts were low to medium polar and soluble in xylene. Therefore, xylene was chosen as primary solvent. Methanol was selected as co-solvent, which is supposed to assist in the formation of emulsion in water during final tank mixture. Butanol was also tried as a

substitute for methanol. The surfactant poly oxyethylene sorbitan monooleate (Tween 20 and Tween 80) was tried in the formulation. The different mixtures of primary solvent, co-solvent and surfactant were prepared initially in varying ratios and the stability of the emulsion in water was tested. No one was found to have required stability. To attain good stability ethylene glycol monoethyl ether as stabilizing agent and silicone anti-foaming agent were added. Finally, six combinations were prepared, out of which the combination-E was finalized on the basis of its quality control assessment (Table 9 and Fig.15). The crude extracts obtained from *P. oxalicum* DWSR1 and *F. oxysporum*DWSR1 were then added in the prepared solvent and adjuvant system (Combination-E) and sonicated for 30 minutes to get two separate 25% (w/v) EC formulation (Fig. 16).

Table 8.Composition of different solvent-adjuvant system

Combination -A:	**Combination -B:**	**Combination-C:**	**Combination-D:**	**Combination-E:**	**Combination F:**
Xylene: 80% Methanol: 10% Glycol: 0 Triton-X-20: 10%	Xylene: 70% Methanol: 10% Glycol: 10% Triton-X-20: 10%	Xylene: 70% Butanol: 10% Glycol: 10% Triton-X-20: 10%	Xylene: 80% Methanol: 10% Glycol: 4.8% Triton-X-80: 5% Silicone antifoaming agent: 0.2%	Xylene: 70% Methanol: 10% Glycol: 9.8% Triton-X-20: 10% Silicone antifoaming agent: 0.2%	Xylene: 70% Butanol: 10% Glycol: 9.8% Triton-X-20: 10% Silicone antifoaming agent: 0.2%

a) Emulsion Stability of the solvent-adjuvant mixtures

EC formulations were evaluated according to CIPAC MT36.1.1 (CIPAC volume F, pl08) at ambient temperature. The volume percent of cream after 0.5, 1 and 24 hours was observed and recorded for a 5 in 100 parts dilution. The emulsion tubes were subsequently inverted 10 times and a final reassessment was made at 24.5 hours. The primary purpose of the emulsion test in this instance is to look for the development of crystals upon dilution. As a result, no effort was made to fully optimize the emulsion performance with respect to cream and oil separation.

Table 9. Emulsion stability study of different solvent-adjuvant combinations

Name of Sample	Ease of dispersion in water	Volume (mL) of creaming off with elapsed time				
		Initial	½ hour	1 hour	24 hours	24.5 hours
A	Excellent	No creamy layer; homogenous emulsion	Trace top cream	1.8 mL cream on top	2.8 mL cream on top	Trace top cream
B	Excellent	No creamy layer; homogenous emulsion	Trace top cream	1.8 mL cream on top	2.6 mL cream on top	Trace top cream
C	Excellent	No creamy layer; homogenous emulsion	Trace top cream	1.8 mL cream on top	2.8 mL cream on top	Trace top cream
D	Excellent	No creamy layer; homogenous emulsion	Trace top cream	1.8 mL cream on top	0.8 mL cream on top	0.4 mL cream on top
E	Excellent	No creamy layer; homogenous emulsion	Nil cream or oil	Nil cream or oil	Nil cream or oil	Nil cream or oil
F	Excellent	No creamy layer; homogenous emulsion	Trace top cream	1.8 mL cream on top	0.8 mL cream on top	0.4 mLcream on top

Fig.15. Combination E; 2: Combination A; 3: Combination D; 4: Combination C; 5: Combination B; 6: Combination F

b) Cold storage stability of the formulation

EC formulations were seeded with at least one crystal of the active ingredient being investigated and stored at 0^0 C for 7 days as per the cold storage stability testing methodology outlined in CIPAC MT39.I (CIPAC Volume F, pl28). On completion of the 7-day storage, formulations were assessed for visible signs of crystal growth. No sediment or crystal was formed

c) Accelerated storage stability of the formulation

EC formulations were stored at 54^0C for 14 days per the accelerated storage stability testing methodology outlined in CIPAC MT46.1.3 (CIPAC Volume F, pl50). On completion of the 14-day storage, the formulations were assessed for stability paying particular note to sedimentation and/or separation.

Appearance	**Formulation of crude metabolites obtained from *P.oxalicum*DWSR1**	**Formulation of crude metabolites obtained from *F.oxysporium*DWSR1**
Initial	Clear, brown, homogenous solution	Clear, brown, homogenous solution
Post-storage (7 days at 0^0C, seeded)	Clear, brown, homogenous solution with crystals. Crystals soluble on thowing*.	Clear, brown, homogenous solution with crystals. Crystals soluble on thowing*.
Post-storage (14 days at 54^0C)	Clear, brown, homogenous solution	Clear, brown, homogenous solution

*Crystals completely re-dissolved on thawing for 1 hour at ambient temperature.

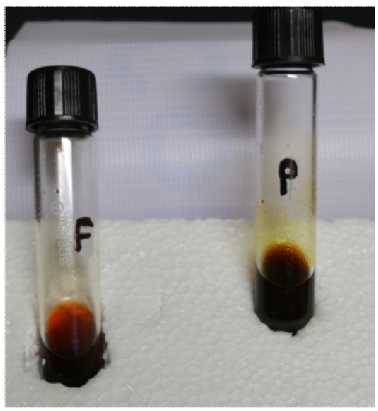


Fig.16. 25% (w/v) EC formulations of *Fusarium* crude extract (F) and *Penicillium* crude extract (P)

The formulated product has been tested for their stability and will be tested under pot culture conditions in the coming season as a follow up of this study.

Summary and Conclusion

Soil antagonistic fungi such as *Fusarium* sp., *Pythium* sp., *Penicillium* sp., *Aspergillus* sp., *Alternaria* spp., were isolated from soil samples collected from the farmers field infected with *Orobanche* in the districts of Neemaj, Gwalior, Bhind, BadraShazipur and Murena of Madhya Pradesh and maintained in pure cultures for further studies in laboratory.According to the result the two fungal bioagents *Fusarium oxysporum* DWSR1 and *Penicillum oxalicum* DWSR1were found to be more effective in delaying the *Orobanche* germination and development when applied as seed treatment and foliar spray after 30 days of sowing and it was found that microbe treated plants had the ability to suppress the haustorial infection from the parasite. The number of haustorial attachments and tubercles were significantly less in the microbe treated plants leading to the increased life of the host plants.

An i*n vitro* germination set up was developed at this laboratory under the project which helps in finding out the germination percentage of to check the viability of *Orobanche* seeds.A simple technique to germinate *Orobanche* in the presence of live host seedlings under controlled conditions is explained here. This technique may be useful in many ways including quantification of the weed seed bank in the infested fields, screening of contaminated seed lots from infested areas for the purpose of quarantine and seed certification. Use of live host seedlings instead of synthetic stimulants, both reduces the cost and makes it possible to use this technique commonly. Limited usage of space, time and money, when compared with other methods for germination of *Orobanche* seeds, makes this technique more advantageous.

Orobanche seeds were identified and collected from different fields of Gwalior infested with *Orobanche* as per their morphological characters given by Plaza et al., 2004 and it was observed that the seeds were dark brown in colour, oblongoid to ovoid in shape, with surface reticulations and weighed 3 to 6 µg. The inflorescence was dense-flowered, corollas usually strongly curved and lobes bluish (Jafri 1978). Flower is pentamerous with violet color at the top and white at base, calyx 4 in number, 9- 12 mm in length, corolla 5 in number, 15-18 mm in length, single pistil of length 16 mm, stamens were 4 in number (8-12 mm length) and styles were 10 mm in length. Based on these characters the species under study was identified as *Orobanche cernua* (Foley, 2004) with

30cm stalk size. According to the results obtained from germination setup it was found that the germinated host plant seedling provides essentially required host signals in a concentrated manner right near the pre-imbibed *Orobanche* seeds, thereby providing a perfect condition for the germination and growth of *Orobanche* seeds. The highest percent viability of *Orobanche* seed was of mustard seed collected from Murena 2 with 67.8% viability.

Secondary metabolite fractions that function as germination stimulants of the seeds of the root parasitic weed were extracted from fungi using dichloromethane and were established to have antagonistic effects on *Orobanche* spp. The metabolite fractions were tested for their efficiency to induce germination, suppress the infection process viz., haustorial attachment, penetration and establishment in the host and induce systemic resistance in mustard against *Orobanche*. Effect of microbial metabolites on the haustorial tissues of *Orobanche* spp. was analyzed using scanning electron microscopic (SEM) by periodically fixing the different stages of infection process using glutaraldehyde which clearly revealed the attachment of the haustoria, haustorial development, infection caused by *Orobanche* and nutrient bridge between the host and *Orobanche* was clearly visible. Systemic resistance was estimated by the production of defense enzymes viz., peroxidase, catalase, phenylalanine ammonia lyase and polyphenol oxidase in the host plants upon allocation of the two fungal bioagents *F.oxysporium* DWSR1and *P.oxalicum* DWSR1, which aid in the defense of the plants during an outside attack on them but the enzymes were not able to delay or develop resistance against *Orobanche* infestation on mustard. On the basis of data collected from pot experiment on mustard and graph plotted it was observed the *F.oxysporium* DWSR1has delayed the *Orobanche* infestation more in comparison to other two bio agents whereas secondary metabolite application of *F.oxysporium* DWSR1and *P.oxalicum* DWSR1 was also effective in delaying *Orobanche* infestations.

Partial purification of the secondary metabolite concentrated from *F. oxysoporum* DWSR1 and *P.oxalicum* DWSR1 extracts were analyzed by LC-MS/MS. Total ion chromatography studies on the different crude metabolites indicated the tentative molecular weight of metabolites of extract of *Fusarium* spp DWSR2 was 447, extract of *F. oxysporum* DWSR1 was 165, 278, 317, 645 and extract of *P.oxalicum DWSR1*: 566, 610, 654.Formulations with six different combinations of solvents and adjuvants were tried in formulating the bioactive crude microbial metabolites obtained separately from *P.oxalicum* DWSR1 and *F.oxysporium* DWSR1. The crude extracts were low to medium polar and soluble in xylene. Therefore, xylene was chosen as primary solvent. Methanol was selected as co-solvent, which is supposed to assist in the formation of emulsion in water during final tank mixture. Butanol was also tried as a substitute for methanol. To attain good stability ethylene

glycol monoethyl ether as stabilizing agent and silicone anti-foaming agent were added. The crude extracts obtained from *P.oxalicum*DWSR1 and *F.oxysporium*DWSR1were then added in the prepared solvent and adjuvant system (Combination-E) and sonicated for 30 minutes to get two separate 25% (w/v) EC formulation. The tests on the Cold storage stability of the formulation indicated that on completion of the 7-day storage there were no sediment or crystal was formed in the formulations. Similarly the Accelerated storage stability test of the formulation indicated that the formulations were stable and there were no sedimentation and/or separation.

Agencies, which can utilize the results of the project.

- MP state Biotech Council
- MP state Agriculture department
- Directorate of Weed Science Research
- Department of Oil seeds

Deliverables for the state of Madhya Pradesh.

- The simple and effective germination setup developed under the project would be helpful in identifying the contaminated seed lots for the purpose of quarantine and seed certification and prevent spread of *Orobanche* into new areas. Further quantification of the weed seed bank in the infested fields is an important step to determine the strategy for management of *Orobanche*. Limited usage of space, time and money, when compared with other methods for germination of *Orobanche* seeds, makes this technique more advantageous.
- The microbes *Penicillium oxalicum* DWSR1and *Fusarium oxysporum* DWSR1 have been identified as potential antagonistic fungi against *Orobanche cernua* in mustard. The metabolite fractions of these fungi have been found to be equally active and they have been formulated in to emulsifiable concentrate formulations. These formulations can be tried at farmer's field levels and depending on their requirement they can be produced and applied in the field. These fungi are easy to mass multiply using simple nutrient media. This method is safe for the environment and can be integrated with any of the management strategies.
- The results obtained in the pathogenicity studies helps in understanding the basics of the host parasite interactions and this information can be used in breeding for resistance against *Orobanche* by Madhya Pradesh state biotech council.

- The information generated in this project may be used by Madhya Pradesh state Agriculture/Horticulture department- for the management of *Orobanche* and increase production

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