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RESEARCH ARTICLE

Molecular and morphological diagnosis of *Orobanche aegyptiaca Pers.* infestation in mustard fields

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ABSTRACT

Orobanche are the devastating holoparasitic weeds causing extensive damage to the mustard cultivation in India. Considering the tedious species identification from a single seed and seed longevity in the soil for years, the mitigation of this weed is difficult. Therefore, development of molecular diagnostic assay specific to *Orobanche aegyptiaca* is required for weed management. In this study, a polymerase chain reaction (PCR) based strategy was optimised using internal transcribed spacer (ITS) based markers to identify the *Orobanche* species predominant in the mustard fields. Genomic DNA was extracted from tissue and soil samples artificially inoculated with seeds of *Orobanche* spp. and subjected to PCR analysis. ITS primers amplified a 350bp PCR product specific to *O. aegyptiaca* confirming its dominance in mustard crop fields of India. Furthermore, soil and tissue samples were collected from the seven farmer fields of Rajasthan and subjected to PCR analysis using ITS-350 primers. ITS-350 primers amplified all the soil/tissue samples confirming the specificity of the method and markers applied. It was also found that one *Orobanche* plant could attach itself to the host plant through many haustoria and also many *Orobanche* plants could attach to the one mustard plant through individual haustorium. This assay can also be applied to identify seed contaminants in commercial seed lots. A small soil sample taken from the mustard field can provide clues about the infestation likely to affect crop yield and productivity. Based on diagnosis suitable recommendations for crop management and input on fertilizer doses can be provided to the farmers on timely basis.

Keywords: Internal Transcribed Spacer (ITS), Molecular diagnosis, *Orobanche aegyptiaca*, Parasitic plant, Rapeseed-Mustard, Soil testing

INTRODUCTION

Over 4000 species of parasitic weeds are major constraint to agricultural production causing heavy damage to various crops thereby reducing both crop yield and quality. Egyptian broomrape or *Orobanche* is an obligate, holoparasitic, phanerogamic, achlorophyllous root parasitic plant that lack chlorophyll and have wider host range including several members of the Solanaceae, Leguminaceae, and Brassicaceae families (Parker and Riches 1993, Wickett *et al*. 2011, Sheoran *et al*. 2014). It attaches itself to the root of mustard plant through haustorium and connects with the host vascular system to derive host water, carbon and nutrients (Schneeweiss 2007) (**Figure 1**). Rapeseed-Mustard is an oilseed crop with a wide range of food and industrial uses and with a major economic significance. In the major mustard cultivating regions of India *i.e*. Haryana, Punjab, northern Rajasthan, western UP and north-east Madhya Pradesh where *Orobanche* (*Orobanche* spp.) has caused enormous menace to mustard production. It is known by various local names such as 'Gudiya' 'margoja', 'khumbhi', 'gulli', 'rukhri', or 'bhuiphod' (Punia *et al*. 2012). Among *Orobanche* spp., *O. aegyptiaca* is dominant weed causing severe yield

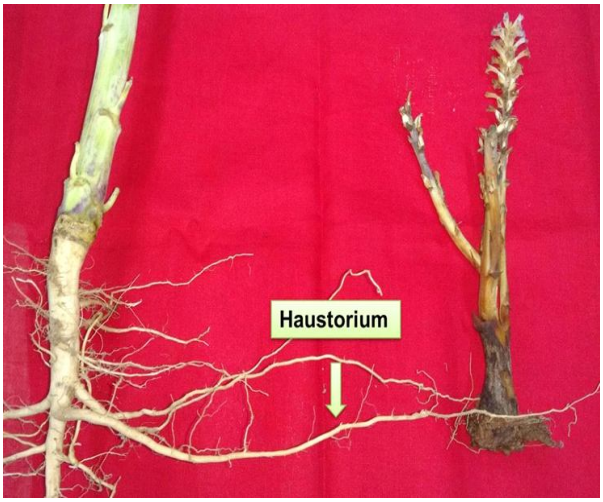

Figure 1. *Orobanche* attached through haustorium on the roots of Indian mustard plant

ICAR-Directorate of Rapeseed-Mustard Research, Sewar, Bharatpur, Rajasthan, 321303, India,

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and quality reduction in rapeseed-mustard. The damage caused by *Orobanche* infestation is often devastating with reported losses up to 28.2% average reduction in Indian mustard yield (Shekhawat *et al.* 2012). It's underground development, miniscule seed size of approximately 180 to 300µm, single plant capacity to produce even over 5 million seeds (against 1,000-odd for mustard), seed longevity for 10-13 years in the soil and appearance of parasite shoots above the soil (70–90 days after sowing) makes its control and detection difficult in the soil and crop seed lot (Jat and Singh 2018). This parasite exerts the greatest damage in the standing crop prior to its emergence causing majority of field loss before diagnosis of infection (Aly 2007, Aly *et al*. 2012) Therefore, identification and classification of *Orobanche* species in a soil sample is very important (Joel *et al*. 1996). So far, the classification of parasitic by taxonomists has been carried out through the morphological features (Hebert and Gregory 2005). However, most of the *Orobanche* species have similar morphological characteristics which, differs in host preference which makes it difficult to classify. In addition, the conventional methods of detection in the soil employ tedious seed separation procedure, observation under binocular microscope and is time demanding (Portnoy *et al.* 1997).

Polymerase chain reaction (PCR) based assays which takes in use of unique DNA such as Internal transcribed sequences (ITS) which are conserved in nature can help in identifying and classifying the *Orobanche* species (Dongo *et al*. 2012). Presently, the tools of biotechnology and molecular biology have revolutionized the way of understanding the knowledge of taxonomy and anatomy to identify plant species with DNA-based marker techniques such as random amplified polymorphic DNA (RAPD) (Katzir *et al.* 1996; Portnoy *et al.* 1997), ITS (Schneeweiss *et al*. 2004; Agarwal *et al.* 2008; Park *et al.* 2008), Inter simple sequence repeat (ISSR) regions (Benharrat *et al.* 2002) and plastid DNA markers (Manen *et al.* 2004; Román *et al.* 2007). Efforts were also made to detect and quantify contamination of *O. aegyptiaca*, *O. ramosa* and *O. Cumana* in soil and crop seed lots of Israel (Dongo *et al*. 2012, Aly *et al*. 2012, Aly *et al*. 2019). Keeping this in view, *O. aegyptiaca* species specific primers were first tested on *Orobanche* spp. affecting the mustard crops cultivated in Rajasthan, India and then molecular diagnosis was performed on the soil collected from the various mustard infested farmers' fields to develop the soil-based diagnosis.

MATERIALS AND METHODS

Sample collection

Orobanche tubercles, shoots, inflorescences, seeds and soil samples used for the experimental purposes were collected from the infested fields of ICAR-Directorate of Rapeseed-Mustard Research in Sewar, Bharatpur, Rajasthan (India) (**Figure 2**) and seven farmers' fields in Bayana district, Rajasthan state, India (26° 54' 0" North, 77° 17' 0" East) *viz.* Seedpur, Bidyari, Nangla Andya, Nangla Jhamra, Sepoura, Vedpura, Bayana villages which represents a typical rainfed site of India. *O. cernua* and *O. ramosa* seeds were also procured for the experimental purposes. *Orobanche* tissue samples were harvested and stored at -80°C while seeds collected from fresh inflorescences were stored at room temperature in dark condition until used. The soil samples collected were taken from the top layer (0–15 cm) of experimental sites.

Soil Sampling and protocol for molecular diagnosis of *Orobanche*:

Sampling location: Soil was randomly collected from the rows within infested mustard field. Since *Orobanche* is attached on the roots of mustard plants,

Figure 2. *Orobanche* infested mustard fields of ICAR-DRMR, Sewar, Rajasthan

90% of seeds fall nearby to it. Therefore, surface and upto 0-8 cm deep soil was taken and pooled for analysis. Soil debris was removed during sampling.

Sampling volume: Around 200g soil was collected from the location and stored at -20°C. However, 100 - 200mg soil sample is enough for DNA isolation and PCR analysis.

Molecular analysis: DNA based analysis to detect seeds of *Orobanche* in soil was performed using ITS-350 primers. Presence or absence of *Orobanche* in soil was confirmed on the basis of 350bp PCR amplification of bands in the samples.

DNA extraction

Genomic DNA extraction from fresh tissues of *Orobanche* spp. and mustard seeds and leaves of DRMR-IJ31 was carried out using modified cetyl trimethyl ammonium bromide (CTAB) method as described according to Doyle and Doyle (1987) with minor modifications. Total Genomic DNA from *Orobanche* soil samples was extracted using the UltraClean soil DNA isolation Kit (MoBio Laboratories, Inc., Solana Beach, CA) according to the manufacturer's instructions. DNA was quantified by analysing on 0.8% agarose gel with λ DNA as standard. The DNA stock solution was adjusted to a concentration of 80-100ng/µl with nuclease free sterile water as the working concentration for the polymerase chain reaction (PCR) and stored at -20° C.

PCR analysis

For PCR analysis ITS-350 primer directed to unique ITS regions corresponding to *O. aegyptiaca* were employed (Aly *et al.* 2012). As a control UCP-555, universal internal control primer was used which amplifies a region of the small subunit of nrDNA (555 bp) from a wide variety of microorganism such as protists, fungi, and plants (**Table 1**). PCR reactions were performed using thermocycler (Eppendorf) in a volume of 20µl PCR mixture containing 100 ng genomic DNA, 1 units of Taq DNA polymerase (G-BIOSCIENCES), 1X PCR Buffer (10mM Tris HCL), 1.mM $MgCl_2$, 100µM each of dNTPs and 10µM of each primer. ITS primers were amplified with following conditions: DNA was denatured at 95°C for 4 min, followed by 35 cycles of 94°C for 1 min each, 52°C for 1 min and 72°C for 1 min, followed by 7 min at 72°C. For amplification with UCP-555 primers, DNA was denatured at 94°C for 5 min, followed by 35 cycles of 94°C for 1 min each, 52.5°C for 1 min and 72°C for 1 min, followed by 7 min at 72°C. Multiplex PCR reaction was performed in 20-µL using the both ITS-350 primers and the universal internal control primers UCP-555. Each reaction tube contained 1X enzyme buffer, 1.5mM $MgCl_2$, 100µM dNTPs, and 0.5µM of the (ITS-350), 0.3µM of the UCP-555 primers, 1.0U *Taq* DNA polymerase, and 2µl (100ng) template (DNA). Amplification parameters were adjusted to 5 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 52°C, and 1 min at 72°C, with a final extension step of 7 min at 72°C (**Table 2**). Amplification products were resolved on 1.5% (w/v) agarose gels in Tris-Borate-EDTA buffer (pH 8.3) and visualized with ethidium bromide under UV light.

Morphology of *Orobanche* plants

Orobanche samples collected from various locations were morphologically classified into length of stalk, number of *Orobanche* shoots attached to one mustard plant, length of haustorium, presence of scales, colour of scales and colour of flowers. Morphological characters of stem, leaf and flowers recorded during the investigation were compared with standard key given by Parker and Riches (1993).

RESULTS AND DISCUSSION

Morphological identification of *Orobanche* species

Morphology helps in the precise identification of species and its management thereafter. However, in case of *Orobanche* which is underground parasitic weed damage on the host plant occurs even before

Table 1. Molecular marker used for the diagnosis of *Orobanche aegyptiaca*

Gene	Primer name	Product sizes	Forward primer (5'-3')	Reverse Primer (5'-3')	References
Internal transcribed spacer	ITS-350	350 bp (+)	CATGGTGGG TGGGGCAACCC	ACGTGATGCGTGACGCCCAG	Aly *et al.* 2012
nrDNA	*UCP-555*	555 bp (+)	GTAGTCATATGCTTGTCTC	GGC TGCTGGCACCAGACTTGC	Aly *et al.* 2012

Table 2. PCR conditions for different primer used for molecular analysis

Primer name	PCR conditions
ITS-350	94° C- 4 min., 94° C- 1 min., 52° C- 1 min. for 35 cycles, 72° C- 1 min., 72° C- 10 min.
UCP-555	94° C- 5 min., 94° C- 1 min., 52.5° C- 2 min. for 40 cycles, 72° C- 1 min., 72° C- 7 min.
Multiplex reaction	94° C- 5 min., 94° C- 1 min., 52° C- 1 min. for 35 cycles, 72° C- 1 min., 72° C- 7 min.

precautionary measures can be taken. Morphological analysis of *Orobanche* samples collected from various mustard fields showed variation in shoot, root and haustoria length (**Figure 3**). It was observed that stem height of *Orobanche* plants ranged from 16-40 cm, mostly branched from the middle point, roundish, yellowish in colour and were thickened at the base. A globular tubercle like structure was present at the base of each stalk acting as reservoir of nutrients for growth and development. *Orobanche* is achlorophyllous plant due to which brown to dark drown scales were present in the raceme manner. Flowers were bisexual, alternate and axially attached to the stem. Cylindrical inflorescence was present; corolla was tubular-infundibuliform, blue-violet, and lighter at the base of tube covered with short hairs outside. Ovary was syncarpous and bicarpellary with terminal single style, 4 numbers of stamens were present in (2+2) manner. The morphological identification described here is in agreement with standard identification key for *Orobanche* species as suggested by Parker and Riches (1993). These findings are also supported by Punia *et al*. (2014) and Jat and Singh (2018) who reported that *O. aegyptiaca* is dominant species affecting mustard cultivation in India and has found similar morphological characteristics for *O. aegyptiaca*. Based on phyto-morphological characters Akhter *et al*. (2020) identified *Orobanche crenata* parasitizing *Cajanus cajan* crop in India. Al-Joboury and Aliwy (2021) also carried out similar morphological studies on *Orobanche* samples collected from Baghdad regions and stated that the average plant height and stem length for *O. aegyptiaca* Pers. is 27.5 and 19.0cm. *Orobanche* affected mustard plants showed shrunken siliquae wilting and yellowing and necrosis of leaves (**Figure 4b** and **c**). However, further studies are needed to assess the extent of damage on mustard plant. In Haryana, Mustard plants infested with *Orobanche* showed wilting symptoms, poor growth and yield (Jat and Singh 2018).

Variation in root architecture of mustard plants collected from farmer fields

Mustard plants attached with *Orobanche* plants collected from different locations showed differences in their root structure. Root architecture of mustard plants (var. DRMR-IJ31) collected from ICAR-Directorate of Rapeseed-Mustard Research, (ICAR-DRMR), Sewar showed well developed tap root system ranging from 25-30cm with non-fibrous adventitious roots while at the other locations root root length of mustard plants ranged from 10-20cm and exhibited well developed adventitious root system

Figure 3a. Showing tubercle, inflorescence, branched stem and root system of fully developed *Orobanche* plant b. brown to dark brown scales in racemose position c. bud stage of *Orobanche* plants d. purple colour tubular-infundibuliform flower e. syncarpus ovary with single style f. four stamens g. seeds forming stage h. roots architecture i. variation in stem length

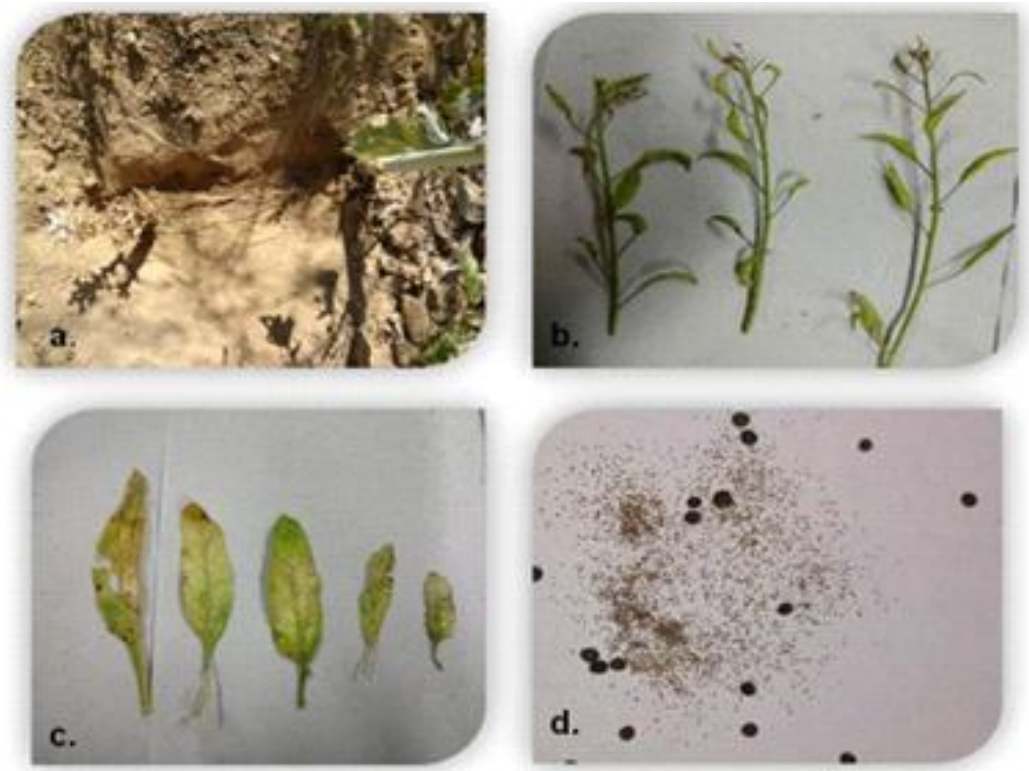

Figure 4a. *Orobanche* attached on the mustard plant through haustorium in mustard crop fields b. & c. silique and leaves of *Orobanche* affected mustard plants d. *Orobanche* and mustard seeds (1.5X)

Table 3. The morphological characteristics of *Orobanche plant* samples collected from various locations

Name of the village	Length of flowering shoot (cm)	Presence and colour of scales	No. of *Orobanche* attached per plant	Colour of corolla	Haustorium length (cm) (Min-Max)	Root (cm) (Min-Max)
Sewar (ICAR-DRMR)	16±5.2	Present (Brown to dark brown)	1-2	violet	2.0-30.0	25.0-30.0
Seedpur	25±3.7	Present (Brown to dark brown)	2-3	violet	3.0-6.5	15.0-20.0
Bidyari	27±4.2	Present (Brown to dark brown)	4-5	violet	4.0-8.0	10.0-16.0
Nangla Andya	30±3.8	Present (Brown to dark brown)	4-5	violet	3.0-10.0	12.0-18.0
Nangla Jhamra	28±5.4	Present (Brown to dark brown)	3-4	violet	1.5-10.0	12.0-16.0
Sepoura	32±3.1	Present (Brown to dark brown)	4-5	violet	6.3-10.0	16.0-18.0
Vedpura	35±4.5	Present (Brown to dark brown)	5-6	violet	1.7-13.0	15.0-20.0
Bayana	28±4.3	Present (Brown to dark brown)	7-8	violet	5.0-10.0	12.0-16.0

in comparison to the main root system with lots of hairy and fibrous structure. In terms of attachment of *Orobanche* to the mustard plants, the length of haustorium was found higher while number of *Orobanche* per mustard plants was lesser in samples collected from Sewar region. In Farmers' field samples, a small haustorium connected *Orobanche* with mustard and higher numbers of *Orobanche* per mustard plants were present. Apart from that, it was found that one *Orobanche* plant can attach itself to the mustard plant through many haustoria and 3-4 *Orobanche* plants can attach to the one mustard plant through individual haustorium (**Figure 5, Table 3**). Soil samples collected from mustard fields of ICAR-DRMR is of clay loam in nature (Shekhawat *et al.* 2012). However, higher sandy and lighter texture was present in soil samples collected from farmers' villages. The soil texture may render it difficult for many *Orobanche* to connect to the host plant but still they were able to penetrate through the roots of host plant. Sandy loam soil is an important soil group of Bharatpur District of Rajasthan. Its texture varies from clays to sandy loam. It is reported that upper profile of sandy laom soil is often deficient in phosphate and calcium while its nitrogen contents vary (Sharma 2019). It is already reported that, *Orobanche* germinates and attaches after receiving certain germination stimulants or strigolactones released by host plant (Westwood *et al.* 2013). Phosphate (P) deficiency promotes strigolactone (SL) biosynthesis in the roots (Yoneyama *et al.* 2012). According to Andreo-Jimenez (2015),

under P limiting conditions, SLs reduces primary root growth, inducing lateral root density and development, and stimulates root hair elongation and density. These modifications allow the plant to increase the exploratory capacity of the soil. This could be possibly one of the reasons why *Orobanche* has better penetration in sandy and clay loam soils. Sheoran *et al*. (2014) also supported the fact that most of the mustard cultivation in India is limited to light textured soil having inherent poor fertility status and water holding capacity which promotes higher germination of *Orobanche*.

Validation of ITS based markers on *Orobanche* samples

Genomic DNA was extracted from the *Orobanche* tissue samples collected from ICAR-DRMR, *O. ramosa* and *O. cernua* seed samples and mustard plants as previously described. Multiplex PCR reaction was carried out to validate the ITS-350 and UCP-555 primers. It was observed that ITS-350 did not amplify the DNA of *O. cernua*, *O. ramosa* and mustard samples. However, the expected 555bp amplicon from the universal primers UCP-555 was present for *O. cernua*, *O. ramosa* and mustard plants, indicating amplification did occur in these samples (**Figure 6**). Furthermore, ITS-350 and UCP-555 primer successfully amplified the 350bp and 555bp PCR product in *Orobanche* samples collected from ICAR-DRMR confirming the abundance of *O. aegyptiaca* in mustard fields of Bharatpur. In a similar study, *Orobanche* samples collected from the oilseed rape infested fields of Israel were confirmed as *O. aegyptiaca* using ITS based markers (Aly *et al*. (2012). In another instance, Osterbauer and Rehms (2002) developed a PCR based assay using ITS based

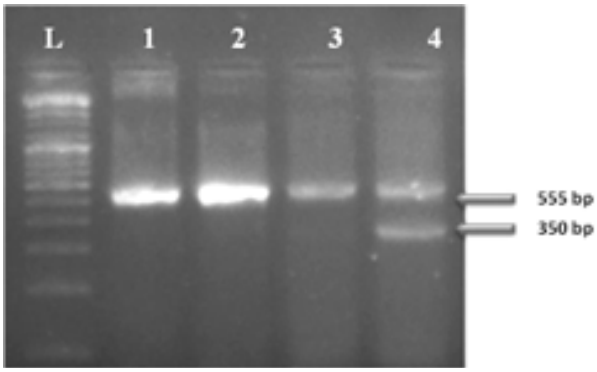

Figure 6. Multiplex PCR demonstrating the specificity of the *Orobanche*-specific primers (350 bp band) and the amplification of the internal control primers (555 bp band). 100bp ladder, Lane1: *Orobanche cernua*, Lane2: *Orobanche ramosa*, Lane 3: Mustard variety (DRMR-IJ31), Lane 4: *Orobanche aegyptiaca*

Figure 5. Variation in *Orobanche* plant samples collected from different mustard farmer fields infested with *Orobanche*

markers to detect *O. minor* seeds from Oregon regions. This assay is sensitive enough to detect a single *O. minor* seed and also not amplify the DNA of red and white clover (*Trifolium pratense* L. and *T. repens* L., respectively), two agricultural hosts for this parasite. Rolland *et al.* (2016) has also developed a high-resolution melting assay (HRM) using plastid markers to identify the eight broomrape species (*O. aegyptiaca*, *O. cernua*, *O. crenata*, *O. cumana*, *O. foetida*, *O. hederae*, *O. minor*, and *O. ramosa*) from a single seed allowing its subsequent use in quarantine purposes in commercial seed lots. Even though, the HRM assay successfully identifies mature plants from the field and seeds in commercial lots but cannot be used to differentiate from each other.

Developing soil-based diagnostic for *O. aegyptiaca*

Soil was collected from a non-infested field (wheat field) in ICAR-DRMR, Bharatpur to develop soil diagnostic assay for *Orobanche*. 200mg of this soil was split into eight samples and artificial infestation of 0, 10, 20, 30, 40, 50, 100, and 150 of *Orobanche* seeds was done. *Orobanche* seeds were collected from the mustard fields in the previous season and stored at room temperature. Total genomic DNA was extracted from the soil samples using DNA isolation kit (UltraClean Soil DNA Isolation Kit, MoBio Laboratories, Inc., Solana Beach, CA) and PCR amplification was carried out using the specific assay designed for this. In this multiplex reaction, ITS-350 and UCP-555 primers successfully amplified a (350bp) and (555bp) PCR product in all the soil samples (**Figure 7**). In Australia, DNA-based soil diagnostics provided assistance in predicting the likely extent of losses from various soilborne diseases caused by fungal and nematode pathogens in wheat and barley crops. Farmers, therefore, have the option of changing cultivars or modifying cropping programs in situations where the risk of crop loss is high (Ophel-Keller *et al.* 2008). In another instance, DNA based soil testing was successfully implemented for quantifying the presence of *P. brassicae* in oilseed rape fields (Wallenhammar *et al.* 2016).

Detecting *O. aegyptiaca* in farmer mustard fields

ITS-350 primers successfully amplified a (350bp) PCR product whereas as expected UCP-555 primers amplified a PCR product (555bp) in all of the soil samples collected from farmer fields. *O. aegyptiaca* infestation was detected in all the soil samples from all the locations which were reported to be infested (**Figure 8**). In one study, Pathak and Kannan (2014) collected soil samples from the

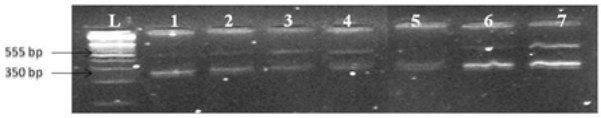

Figure 7. Multiplex PCR showing PCR amplification of *Orobanche* DNA isolated from soil containing *Orobanche* seeds. *Orobanche* specific primers amplified desired 350bp product whereas Universal primer gave 555bp product. 100 bp ladder, L: soil containing 10 seed of *Orobanche*, Lane 1: 20 seeds of *Orobanche*, Lane 2: 30 seeds of *Orobanche*, Lane 3: 40 seeds of *Orobanche*, Lane 4: 50 seeds of *Orobanche*, Lane 5: 100 seeds of *Orobanche*, Lane 6: 150 seeds of *Orobanche*, Lane 7

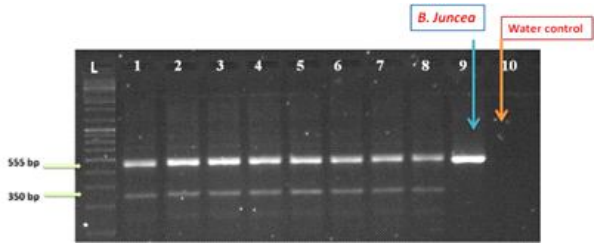

Figure 8. Multiplex PCR demonstrating the specificity of the *Orobanche aegyptiaca*-specific primers (350 bp band) and the amplification of the internal control primers (555 bp band). *Orobanche* samples from village: Bidyari, Seedpur, Nangla Andua, Seopura, Vedpura, Sewar, Bayana, Nangla Jhamra, Rajasthan, India (lanes 1 -8), *Brassica Juncea* (lane 9), and water control (lane 10) and 100 bp DNA ladder (L).

tomato and mustard farmer fields of Gwalior regions of Madhya Pradesh and quantified *Orobanche* seed bank with viability on host crops such as mustard, brinjal and tomato. On the basis of morphological characters *Orobanche cernua* was identified as the invading species. The ITS region is conserved in nature and ITS markers has the potential to identity underground plants with greater accuracy (Linder *et al.* 2000). Therefore, molecular based assay has edge over the morphological identification for resolving the identity of closely linked species. Aly *et al.* (2019) quantified the number of *Orobanche* seeds present in a soil sample from a sunflower-infected field using ITS-100bp markers. Kirilova *et al.* (2019) also identified seeds of *O. ramosa*, *O. mutelii* and *O. cumana* in the soils collected from different farmer fields in Bulgaria by ITS based molecular markers.

In this study, the molecular and morphological diagnosis of *Orobanche* was carried out to detect the prevalent species and presence of seeds in mustard fields. The use of this technique on soils collected from farmer's fields of Rajasthan showed that *O. aegyptiaca* is major dominant species affecting

mustard crop as its DNA was detected in 100% of the eight fields sampled. Thus, a high risk of complete crop failures exists if a susceptible mustard cultivar is sown by farmers in Rajasthan, India. Jat and Meena (2018) reported control techniques for *Orobanche* infestation in mustard fields at Dausa district of Rajasthan which farmers can utilise.

In conclusion, DNA based soil testing can be applied for early detection of *Orobanche* seed bank in soil which can lower the risk of crop yield loss and helps in maintaining plant and soil health. Furthermore, identification of parasitic weed seeds in crop seed lot before a crop is planted can allow cultivators to take precautionary measures and prevent further spreading of this weed to parasite free fields.

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