Mycopath (2012) 10(2)**:** 63-66 **Research Article**

In vitro screening methods using chemical fungicides against canola black spot pathogen

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Abstract

Canola is a cash commercial crop renowned for its edible oil production ability. Brown and black spot diseases of stem and leaves of canola plants caused by *Alternaria* species have always been a threat for their growth and productivity ultimately economic loss. Several strategies have been adopted to control these fungal pathogens, while, use of commercially available chemical fungicides is the most common. For this in present study, two chemical fungicides with trade name Triton and Benedict contains active ingredients validamycin and iprobenfos, respectively were evaluated for their ability to control the canola spot disease pathogen, *Alternaria* sp. Fungus was grown on growth media incorporated with fungicides by three different methods viz. well diffusion, disc diffusion and food poisoning. Maximum inhibition in fungus growth was recorded by food poisoning method using either of fungicide. Whereas, Benedict (iprobenfos) was more effective against *Alternaria* sp. as compared to Triton (validamycin). Therefore, this method is suggested for researchers being more efficient for laboratory assays.
Keywords: *Alternaria*, canola, chemical control, fungicides.

Introduction

Alternaria leaf/stem black spot disease is a threat to canola, an economically important crop. The reported causal species are *Alternaria alternata, A. brassicae* and *A. raphani* and their disease severity is related to moisture and temperature conditions. *Alternaria* spots are found at every growth stage of canola plants, however mature plants are more susceptible than young plants. Pathogens infect their hosts by direct entry through wounds or natural openings. The disease is favoured by warm and humid weather. Disease is spread by pathogen conidia within and among fields by water and wind. The pathogens survive in infested crop waste and on seed (Conn *et al*., 1990).

Several management strategies are available to control black spot disease of canola like manual, mechanical and cultural methods (Schwartz and Gent, 2004). One of the most effective and old method for disease control is the use of chemical fungicides. There are several fungicides which are being commercially available, while several others are being evaluated in different laboratories. Seidle *et al*. (1995) found that *Alternaria* infection generally increased on green seed of *B. rapa*, caused seed shrivelling and substantial yield reductions in crop. Fungicides, particularly foliar spraying with Rovral (iprodione) at late flowering, reduced yield losses, seed infection and increased seed germination rates. The combination of Emisan-6 with Indofil M-45 was found to be most effective followed by the combination of Emisan-6 with Indofil Z-78 in controlling *Alternaria* blight of potato (Singh *et al*., 1997). Mancozeb followed by Captafol were reported very effective against *A. solani* infecting tomato plants (Babu *et al*., 2000). Iprodione and mancozeb were documented as effective systemic fungicides against *Alterneria* disease (Parsad, 2002).

The objective of this present study was to evaluate and compare the inhibitory efficacy of two most commonly used fungicides, Triton (validamycin) and Benedict (iprobenfos) using laboratory assay techniques against *Alternaria* sp.

Materials and Methods

Two fungicides namely Triton and Benedict contain validamycin and iprobenfos, respectively as active ingredient were procured from the Kanzo Ag (Evyol Group), Lahore, Pakistan. These fungicides were evaluated for their potential to control the pathogen, *Alternaria* sp. grown under laboratory conditions, using three different techniques as follows.

Disc diffusion method

In disc diffusion method, spores (10^5) of *Alternaria* sp. were distributed uniformly under aseptic conditions onto the surface of MEA (malt extract agar). Then a disc of 6 mm diameter was cut from the pre sterilized Whatman filter paper

No 1, saturated with recommended dose of the fungicide and placed on agar plates. These Petri plates were first refrigerated for 2 hours to allow diffusion of the compounds presents in the fungicides into the growth medium and then incubated at 25 °C ± 2 for 5 days. Sterilized water was used as control. Growth inhibition was assessed by the presence of opaque halo around the disc of filter paper. Diameter of inhibition zone (cm) formed on agar plate was measured and compared with control (De Billerbeck, 2007).

Agar-well diffusion method

Qualitative antifungal screening was carried out using the agar-well diffusion method as described by Shirurkar *et al*. (2012). Fungal spores (10^5) were spread thoroughly as described in the previous method. A 6 mm well was created in the centre of each medium plate using the sterilized cork borer. Recommended doses of various fungicides were poured into the wells. The plates were incubated for 5 days at 25 °C ± 2. Antifungal activity of fungicides was evaluated by measuring zone of no growth (cm). In control treatments, sterlized water was used instead of fungicides. Percentage inhibition was calculated using the following formula.

$$\text{MGI (\%)} = \frac{\text{DC - DT}}{\text{DT}} \times 100$$

DT = diameter of the fungal colony (cm) in the blank Petri dish; DC = diameter of the fungal colony (cm) on fungicide treated growth medium.

Food poisoning method

Hyphal growth inhibition on the growth medium incorporated with fungicide was determined according to method described by Ngono *et al.* (2000). Calculated dose of each fungicide was added to sterile molten approximately 25 mL of MEA (at 45 ºC). After mixing thoroughly, medium was poured in the pre-sterilized Petri plate. Control treatments received an equivalent amount of sterilized water. A plug of 3 mm of fungal mycelium was cut from the edge of actively growing fungal colony and placed in the center of agar plate. All treatments were incubated for 5 days at 25°C ± 2. Radial fungal growth was measured and inhibition was calculated using the same formula as described for the food poisoning method.

Duncan's Multiple Range test ($P \leq 0.05$) was used to delineate treatment mean (Steel and Torrie, 1980), using computer software COSTAT.

Results and Discussion

Alternaria spot diseases are very common problem in agriculture (Akhtar *et al.,* 1994). Based on previous findings (Mesta *et al*., 2011; Arain *et al*., 2012), it is believed that chemical fungicides are best option to control *Alternaria* spot diseases. During present study, antifungal potential of two commercially available fungicides with trade name Triton (validamycin) and Benedict (iprobenfos) were evaluated against canola leaf spot pathogen (*Alternaria* sp.) by three methods i.e. well diffusion, disc diffusion and food poisoning method. Zone of no growth was used as parameter to study the inhibition efficiency of each fungicide.

Generally, both fungicides significantly inhibited the fungus growth by food poisoning method as compared to well diffusion and disc diffusion methods. Whereas, Benedict (iprobenfos) was more effective against *Alternaria* sp. as compared to Triton (validamycin).

The fungus growth was significantly inhibited by 71.29% with maximum zone of inhibition due to Benedict using food poisoning technique as compared to control. In contrast to that, fungal growth was inhibited by 27% and 10% using well diffusion and disc diffusion methods, respectively with less zone of inhibition over respective control treatments (Fig. 1 & 2, Table 1).

Triton depicted its low level efficacy using either method. Therefore, fungus growth was inhibited within range of 10-21% with three different methods (Fig. 1 & 2, Table 1). Maximum growth inhibition with food poisoning method is attributed to complete mixing of fungicide with agar medium along with uniform concentration everywhere (Ngono *et al.*, 2000). In other two methods, fungicide was present in the centre and possibly diffused out to neighboring media, therefore is unlikely no fungicide at the periphery of the medium plates.

Benedict was found to be more effective against *Alternaria* sp. as compared to Triton. Iprobenfos, the active ingredient of Benedict is a key compound that is available in different commercial names. Being organophosphorous in nature, this compound has a long history to control the various fungal diseases including *Alternaria* spp. Its antifungal activity is well-correlated with hindrance in the synthesis of phospholipid and methytransferase in fungi (FRAC, 2006). Srivastava and Mishra (2008) documented considerable growth inhibition of *Colletotrichum truncatum* by iprobenfos. Although the control by Triton that have validamycin as active ingredient

is less as compared to Benedict, however it also has a wide use in inhibiting the fungal pathogens for example rice sheath blast (He *et al.*, 2003). The chemical family of validamycin is lucopyranosyl. The mode of action of validomycin is to inhibit trehalase and inositol biosynthesis in fungi (FRAC, 2006). Currently, low antifungal activity could be attributed its non-specificity against the *Alternaria* sp.

Food poisoning method was found to be effective technique to assess antifungal activity of chemical fungicides against leaf spot pathogen of canola i.e. *Alternaria* sp. as compared to well diffusion and disc diffusion methods. Benedict (iprobenfos) exhibited significant potential to inhibit growth of *Alternaria* sp. in comparison to Triton (validamycin).

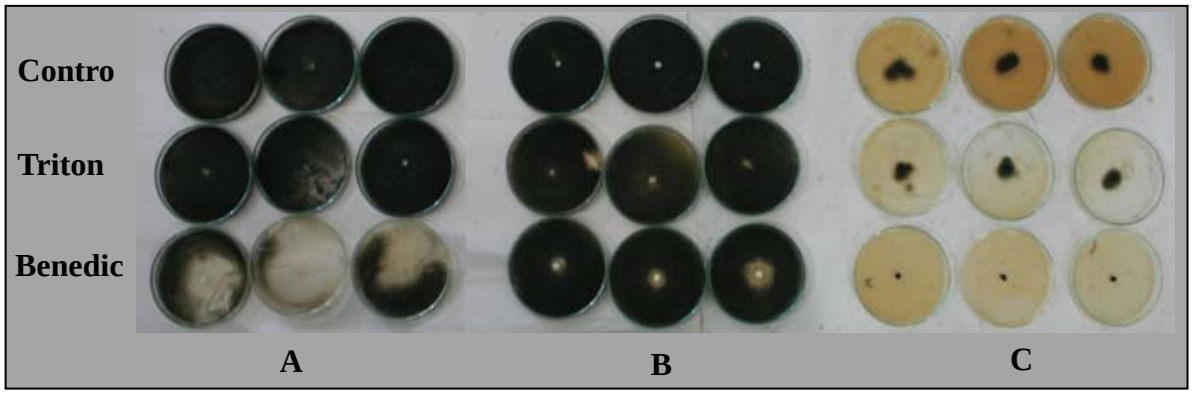

Fig. 1: *In vitro* efficiency of screening methods to evaluate the antifungal potential of Triton and Benedict.

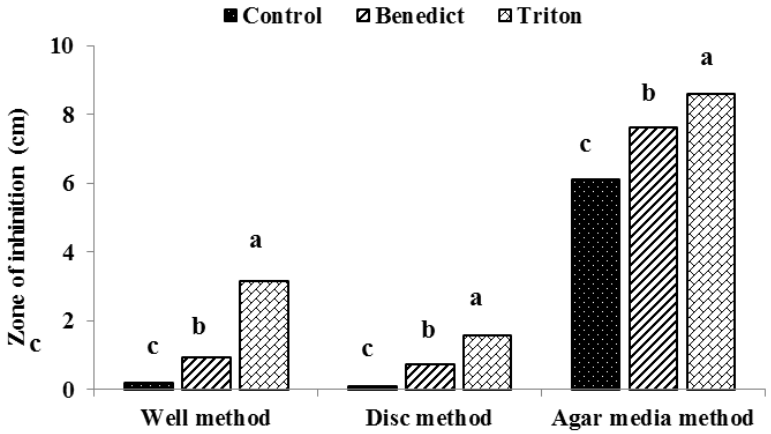

Fig. 2: *In vitro* efficiency of screening methods to evaluate the antifungal potential of Triton and Benedict.

Vertical bars show standard errors of means of three replicates. Values with different letters show significant difference (P≤0.05) as determined by Duncan's Multiple Range Test.

Table 1: Percentage of growth inhibition by Triton (validamycin) and Benedict (iprobenfos).

Fungicide	Growth inhibition (%)		
	Well diffusion method	Disc diffusion method	Food poisoning method
Validamycin	10.37 d	8.14 e	21.28 c
Iprobenfos	27.27 b	10.08 d	71.29 a

Values with different letters show significant difference (P≤0.05) as determined by Duncan's Multiple Range Test.

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