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Efficacy of Fungicides and Bio Agents in Managing the Black Leaf Spot Disease of Cabbage caused by *Alternaria brassicicola* (Schw.) Wiltsh

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ABSTRACT: Black leaf spot caused by *Alternaria brassicicola* is one of the destructive diseases of crucifers and causes considerable loss in terms of quantity and quality. The yield loss due to Alternaria was 5-30% in the entire cabbage growing areas of India. Hence, experiments were conducted to determine the efficacy of fungicides and bioagents both *in vitro* and *in-vivo* against *A. brassicicola* causing black leaf spot of cabbage. *In vitro* studies were carried out by using poisoned food technique in case of fungicides and dual culture technique for bioagents in completely randomized design. All the fungicides and bioagents significantly inhibited the mycelial growth. Among the test fungicides; hexaconazole, difenconazole and propiconazole @1000ppm; chlorothalonil @2000ppm and mancozeb @ 2500ppm were found significantly effective and inhibited the mycelial growth cent per cent. Among the bioagents; *T. viride* recorded significantly highest level of inhibition (76.84%) and caused lysis and mycoparasitism on *A. brassicicola* after 20 days of incubation. The treatments which were found effective under laboratory conditions were evaluated at field level by foliar application of each treatment at 15 days intervals for three times. Triazole compounds like hexaconazole, difenconazole and propiconazole reduced disease severity by 49.04, 41.81 and 35.87% and increased yield by 24.50, 19.71 and 12.57%, respectively. Hence, such effective fungicides could be used to minimize disease severity. Significant effect of some bioagents against pathogen growth suggests their application as alternatives to chemicals.

Keywords: *Alternaria brassicicola*, Bioagents, Cabbage, Hexaconazole, *T. viride.*

INTRODUCTION

The vegetables belonging to genus *Brassica* are often referred to as "cole crops" and comprises of cabbage, cauliflower, broccoli, kales, kohlrabi, *etc*. Cabbage (*B. oleracea* L. var. *capitata*) is grown as annual vegetable crop for its compact head which varies in shape from flat to long-oval out line (Nieuwhof, 1969).It was originated from a wild species *B. oleracea* var. *oleracea* (*sylvestris* L.), commonly known as wild cabbage or 'Colewort' (Balliu, 2014).

Cabbage production is affected by many fungal, bacterial and viral diseases at different stages of growth and development. Among fungal diseases; black leaf spot of cabbage may be caused by different *Alternaria* species *viz.A. brassicicola* (Schw.) Wiltsh., *A. brassicae* (Berk.) Sacc., *A. alternata* (Fr.) Kreissler and *A. raphani* (Groves and Skolko); (Kumar *et al*., 2014) and these casual organisms are responsible for significant yield losses (Verma and Saharan 1994) by reducing photosynthetic activity, accelerating senescence, defoliation, premature pod shatter and shriveled seed leading to considerable reduction in quality and quantity of yield products (Shresta *et al*., 2000; Kumar *et al*., 2014). The yield loss due to Alternaria was 5-30% in the entire cabbage growing areas of India (Pandey *et al*., 2002). Due tono proven source of resistance reported till date in any of the hosts (Meena *et al*., 2012) and also by continuous availability inoculum from numerous sources like seed, infected plant debris, collateral hosts *etc*., and wide range of spore dispersal (King, 1994), this disease is very difficult to manage. The use of fungicides is the most popular method for management of black leaf spot disease of cabbage. Hence, the present investigations were made *in vitro* and *in vivo* to find out the effective fungicide(s) and bioagents (s) against *A. brassicicola* causing black leaf spot disease of cabbage.

MATERIAL AND METHODS

The experiments were carried out at Department of Plant Pathology as well as Instructional farm of College of Horticulture, Venkataramannagudem. Black leaf spot infected leaf samples were collected from farmer's field and observed for the presence of the pathogen

Alternaria. After assuring the presence of *Alternaria* spores, standard tissue isolation technique was followed for isolation from infected leaf tissues. Pathogen was purified by single spore isolation method and identified as *A. brassicicola* based on cultural, morphological and molecular characters by sequencing the ITS regions. The ITS sequence was deposited in NCBI GenBank and obtained accession number is OP161144.

A. In vitro screening of fungal and bacterial bio-agents against A. brassicicola

In vitro* efficacy of fungicides against *A. brassicicola. *In vitro* efficacy of seven fungicides *viz*., difenconazole 25% EC, hexaconazole 5% EC and propiconazole 25% EC @1000 ppm; captan 70% + hexaconazole 5% WP @ 1500 ppm; iprodione 50% WP and chlorothalonil 75%WP @2000 ppm and mancozeb 75% WP @ 2500 ppm was evaluated against *A. brassicicola* by poisoned food technique (Nene and Thapliyal 1993) on PDA medium. Requisite quantities of fungicides were calculated and solutions were prepared based on active ingredient, which were then added separately to the autoclaved, cooled (40°C) PDA in conical flasks just before pouring into Petri plates. Each poisoned PDA media was poured aseptically in Petri plates (90 mm dia.). After solidification, all the plates were inoculated aseptically and separately with five mm culture disc of *A. brassicicola* obtained from seven days old actively growing edges of pure culture placed at the centre of Petri plates. Petri plates containing plain PDA (without fungicide) inoculated with the test pathogen served as control. All the plates were incubated at 25±1°C. The experiment was designed in Completely Randomized Design (CRD) and all the treatments were replicated thrice. Observations of radial mycelial growth were recorded in all the treatment plates. Per cent inhibition of mycelial growth was calculated whenever Petri plate full growth of the test pathogen was observed in control plate by using the formula given by Vincent (1947).

$$\text{Per cent inhibition} = \frac{C - T}{C} \times 100$$

Where,
C = Diameter of fungal growth in control (mm)
T = Diameter of fungal growth in treatment (mm)

In vitro* bio efficacy of bioagents against *A. brassicicola. *In vitro* evaluation of bio efficacy of bio-agents was carried out in CRD with seven treatments; containing four fungal (*Trichoderma harzianum, T. viride*, *T. reesei* and *T. koningii*) and two bacterial (*Pseudomonas fluorescens* and *Bacillus subtilis*) bioagents in three replications on PDA medium using dual culture method (Morton and Stroude 1955). Twenty ml of sterilised and cooled PDA medium was poured aseptically into 90 mm Petri plates and left to solidification. Five mm mycelial discs from the edges of seven days old culture of *A. brassicicola* as well as from fungal bioagents with the help of sterile cork borer were placed in opposite directions over the solidified PDA in such a way that the distance between each other was approximately 80 mm. In case of bacterial bioagents, the test pathogen was placed at one end of the Petri dish and pure cultures of bacterial bioagents were streaked with sterile inoculation loop at the centre. Control was maintained by inoculating *A. brassicicola* at the centre of Petri plate. The inoculated Petri plates were incubated in an incubator at 25 ± 1°C. Antagonistic activity, zone of inhibition and nature of parasitism were recorded. Per cent inhibition was calculated using the formula of Vincent (1947).

B. In vivo evaluation of fungicides and bioagents

The fungicides and bioagents that showed effective growth inhibition under *in vitro* were selected to assess their efficacy under field conditions during *Rabi* crop season of 2021-2022 at Experimental plots of COH, V.R Gudem. Experiment was laid out in Randomized Block Design (RBD) with three replications and nine treatments including control. Five fungicides *viz*., propiconazole, hexaconazole, difenconazole @0.1%; mancozeb @0.25; chlorothalonil @0.2%; two fungal and one bacterial bioagents *viz*., *T. viride*, *T. harzianum*@10^6 cfu/ml and *B. subtilis* 10^8 cfu/ml were used.

Field preparation was carried out during the first week of October 2021-2022 and experimental block was divided into 27 plots and twenty one days old seedlings of cabbage (Golden boll) were transplanted at a spacing of 60 × 45 cm separately on 15th October 2021 at optimum soil moisture level. Each treatment was maintained in three rows consisting of 15 plants per row. All the recommended practices for cultivation of cabbage were followed as per the package of practices of Dr YSR Horticultural University, Andhra Pradesh in order to raise healthy crop.

Spore suspension of *A. brassicicola* containing inoculum load of 1 × 10^6 spores/ ml was sprayed over the foliage at 25 days after transplanting (DAT) on cabbage (Sailaja *et al.,* 2017). First spray of the treatments was done immediately after appearance of disease symptoms on lower and upper leaves and subsequently two sprays were followed at 15 days interval between sprays. Disease severity was assessed on 35, 50 and 65 DAT using 0-5 scale (0= no infection, 1= less than 5% leaf area infected, 2= 5-10% leaf area infected, 3= 10-25% leaf area infected, 4= 25-50% leaf area infected, 5= more than 50% leaf area infected (Sangeetha and Siddaramaiah 2007). Ten plants were selected randomly per each treatment, for disease assessment. Per cent Disease Index (PDI) by McKinney (1923) and Area under Disease Progress Curve (AUDPC) by Jerger (2004) were calculated for all the treatments. Heads were harvested separately for each treatment after attaining the maturity and yield of net plot was recorded in terms of kg and later expressed in t/ ha.

$$\text{Per cent disease index (PDI)} = \frac{\text{Sum of all ratings}}{\text{Total number of plants observed} \times \text{Maximum disease rating scale}} \times 100$$

$$\text{AUPDC} = \frac{[\{(X_1 + X_2)/(2)\} + \{(X_2 + X_3)/(2)\}] \times D}{n - 1}$$

Where
X= Per cent disease
Index at different dates (D_1, D_2, D_3 and so on)
D= Time interval between two observations (days)
N= Total number of observations

RESULTS AND DISCUSSION

A. In vitro evaluation of fungicides and bioagents against A. brassicicola

Evaluation of fungicides against *A. brassicicola.* A total of eight treatments with seven fungicides *viz.,* hexaconazole, difenconazole and propiconazole @ 1000 ppm, captan + hexaconazole @1500ppm, chlorothalonil and iprodione @2000ppm and mancozeb@ 2500ppm were evaluated under *in vitro* conditions against *A. brassicicola* by poisoned food technique. All the tested fungicides significantly inhibited the mycelial growth over the control and the results presented in Table 1 reveals that each fungicide at specified concentration showed varied levels of per cent inhibition.

Among the fungicides tested, cent per cent inhibition of mycelial growth of *A. brassicicola* was observed in hexaconazole, propiconazole, difenconazole @1000ppm, chlorothalonil @2000ppm and mancozeb @2500ppm. The next best treatment in inhibiting the mycelial growth was captan + hexaconazole @1500ppm (75.89%) and the lowest per cent inhibition (30.46%) was shown by iprodione @2000ppm (Fig. 1). The triazole group of fungicides like hexaconazole, propiconazole, difenconazoleact on one specific enzyme *i.e.* C14- demethylase, which plays a key role in sterol biosynthesis. Sterols such as ergosterol is essential for the cell membrane structure and function. It is essential for the development of functional cell walls. Mancozeb being multisite inhibitor effects lipid metabolism, respiration and production of ATP and interferes with enzymes containing sulphydryl groups, disrupting different biochemical process within the fungal cell cytoplasm and mitochondria.

Table 1: *In vitro* evaluation of fungicides against *A. brassicicola.*

Treatments	Conc.(ppm)	Cabbage isolate	
		Mean mycelial growth (mm)	Per cent inhibition
Hexaconazole	1000	0.00*	100.00 (90.00)**
Captan + Hexaconazole	1500	21.69	75.89 (60.57)
Iprodione	2000	62.58	30.46 (33.48)
Mancozeb	2500	0.00	100.00 (90.00)
Propiconazole	1000	0.00	100.00 (90.00)
Chlorothalonil	2000	0.00	100.00 (90.00)
Difenconazole	1000	0.00	100.00 (90.00)
Control		90.00	
CD at 1%			0.51
SE.m (±)			0.25
C.V (%)			0.65

* Mean of three replications **Figures in parentheses are transformed values

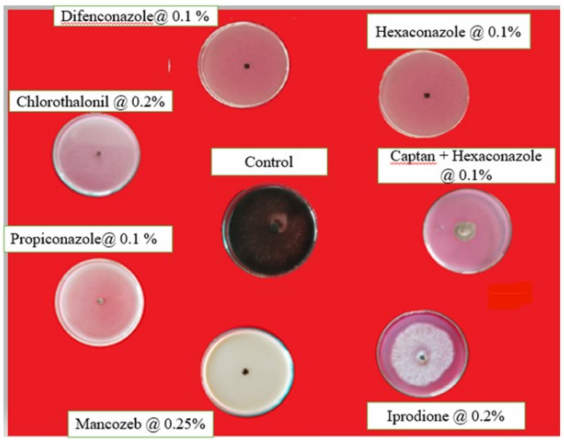

Fig. 1. *In vitro* efficacy of fungicides against *A. brassicicola.*

These results are in accordance with the findings of several earlier workers. Fungicides *viz*., propiconazole, difenconazole and hexaconazole used by Hossain and Main (2004); Gaikwad (2013); Tu *et al.* (2015), Pratima *et al*. (2017), mancozeb by Singh *et al.* (2017) and chlorothalonil by Tu and Somasekhara (2015) on inhibition of mycelial growth of *A. brassicicola*.

Bio efficacy of bioagents against *A. brassicicola.* Seven treatments comprising of four fungal bioagents, two bacterial bioagents and one untreated control were evaluated by using dual culture technique against *A. brassicicola.* Results presented in Table 2 and Fig 2. revealed the significant effect on mycelial growth inhibition. The bio agents inhibit the growth of the

pathogen either by over growing or by formation of inhibition zone. Evaluations against *A. brassicicola* showed that *T. viride* was highly efficient by inhibiting (76.84%) of mycelial growth, followed by *T. harzianum* (64.90%) and both were significantly different with each other. The lowest inhibition was shown by *T. reesei* (31.24%). The growth inhibition noticed in case of bacterial bio agents were; *B. subtilis* (58.56%) and *P. fluorescens* (52.87%). The fungal bioagents showed mycoparasitism by causes the lysis of mycelium of *A. brassicicola* after 20 days of incubation (Fig. 3).

Table 2: *In vitro* bio efficacy of bio-control agents against *A. brassicicola.*

Treatments	Mean mycelial growth (mm)	Per centinhibition
Pseudomonas fluorescens	42.41*	52.87(46.63)**
Bacillus subtilis	37.29	58.56(49.91)
Trichoderma viride	20.83	76.84 (61.21)
Trichoderma harzianum	31.59	64.90 (53.65)
Trichoderma reesei	61.87	31.24 (33.97)
Trichoderma koningii	54.78	39.13 (38.71)
Control	90.00	
CD at 1%		2.20
SE.m (±)		0.72
C.V (%)		2.58

* Mean of three replications **Figures in parentheses are transformed values

Fig. 2. *In vitro* efficacy of bioagents against *A. brassicicola.*

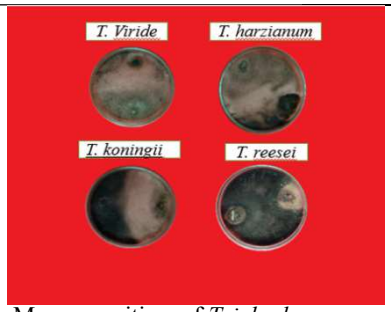

Fig. 3. Mycoparasitism of *Trichoderma* spp. on *A. brassiscicola* after 20 days ofincubation.

The antagonistic activity of *Trichoderma* spp. is mainly due to antibiosis by production of volatile components and non-volatile antibiotics could be possible cause of antagonism, competition for nutrients and niche competitions and also by the secretion of extracellular cell degrading enzymes such as chitinase, β-1,3-glucanase, cellulose, lectin and other secondary metabolites such as glioviridin, viridin and gliotoxin which may help mycoparasites in colonization of host. The results obtained were in line with the findings of Ahmad and Ashraf (2016), who found the bio efficacy of *T. viride, T. harzianum, T. hamatum, T. koningii, T. reesei* on growth inhibition. Similar findings were noticed by Jackson and Kumar (2019) and Pun *et al* (2020) on *A. brassicicola.*

Mycelial growth inhibition of *A. brassicicola* by *P. fluorescens* and *B. subtilis* through direct antagonism of phytopathogens and siderophores was observed. These results are in accordance with Khalse *et al*. (2017).

B. In vivo evaluation of effective fungicides and bioagents against black spot disease

On the basis of *in vitro* antifungal activity of fungicides and bio-agents, effective treatments were selected and applied as foliar sprays at 15 days intervals. The results obtained by applying various treatments in managing black leaf spot disease severity, AUPDC and yields were recorded and presented in Table 3.

Table 3: Effect of fungicides and bioagents against black leaf spot of cabbage under field conditions.

Treatments	TPDI	Per cent reduction over control	AUDPC	Yield (t/ha)	Per cent increase in yield over control	Relative yield loss (%)
Hexaconazole	37.41*(37.69)**	49.04	418.90	17.43	24.50	
Mancozeb	54.57 (47.60)	25.66	676.35	15.30	9.29	12.22
Propiconazole	47.08 (43.31)	35.87	563.95	15.76	12.57	9.58
Chlorothalonil	53.96 (47.25)	26.50	667.2	15.20	8.57	12.79
Difenconazole	42.72 (40.80)	41.81	498.55	16.76	19.71	3.84
Trichoderma harzianum	61.48 (51.62)	16.25	779.95	14.90	6.43	14.52
Trichoderma viride	63.21 (52.64)	13.89	806.40	14.73	5.21	15.49
Bacillus subtilis	68.66 (55.94)	6.47	887.60	14.56	4.00	16.47
Control	73.41 (58.94)		958.90	14.00		19.68
CD at 5%	3.22			1.91		
SEm (±)	1.06			0.63		
C.V(%)	3.30			0.71		

*Mean of three replications **Figures in parentheses are transformed values

A total of three spraying schedules of each treatment was planned. The first spraying was given 30 DAT. Data obtained revealed that all the treatments numerically influenced the per cent disease index of black leaf spot and significantly effective in managing the disease. The disease was found to be appeared at about 30 DAT and later increased steadily up to second spraying and slightly less increase was observed thereafter. Per cent disease index showed significant difference between sprayed and un-sprayed plots at 65 DAT (Terminal PDI).

From the Table 3, it was evident that all the treatment options were significantly superior over control and reduced the per cent disease index. The terminal PDI ranged from 37.41 to 68.66%, while 73.41% was observed in control plot. Among the treatments, hexaconazole and difenconazole were significantly superior over rest of the treatment and controlled the disease by recording lowest PDI values of 37.41 and 42.72%, respectively and were at par with each other. The next best treatment in controlling the black leaf spot was propiconazole with PDI of 47.08%andwas significantly superior to the rest of the fungicides and bioagents. In case of bioagents, PDI ranged from 61.48 to 68.66%. Lowest PDI was observed in *T. harzianum* sprayed plot whereas the highest PDI was observed in *B. subtilis,* which significantly differ with each other.

The relative influence of disease development was assessed by AUDPC. The mean severity was used to calculate the AUDPC and results were furnished in Table 3. The increase in disease severity throughout the assessment days indicated the spread of disease in space and time. The data showed variation in spread of disease among fungicides. Highest AUDPC values 958.90%-days were observed from the control plots, whereas, the lowest AUDPC values 418.90%-days and 498.55%-days were recorded from hexaconazole and difenconazole sprayed plots, respectively. Similarly in case of bioagents, lowest and highest AUPDC was recorded in *T. harzianum* (779.95%-days) and *B. subtilis* (887.60%-days), respectively.

With respect to reduction of disease over control, among the fungicides; hexaconazole showed superiority with highest reduction of disease (49.04%). This was followed by difenconazole (41.81%) and propiconazole (35.87%). Among the bio agents; *T. harzianum* was effective in reducing the disease severity (16.25%) followed by *T. viride* (13.89%).

The results of field experiment (Table 3) on cabbage revealed that, all the treatments were significantly effective and reduced the disease severity and there by increased the yield compared to untreated check (control). Significant differences were found among the treatments regarding the efficacy of fungicides and bio-agents on yields. Yield (t/ha) ranged from 14.56 to 17.43 in other treatments and 14.00t/ha was recorded in control plot. Plots that were sprayed with hexaconazole, difenconazole and propiconazole recorded significantly highest yields *viz.*, 17.43, 16.76 and 15.76 t/ha, respectively and these three treatments were at par with each other and the remaining treatments both fungicides and bioagents were also at par with each other.

The computed relative yield losses showed notable differences among treatments. Yield losses were highly reduced by fungicide sprayed plots as compared to the bio-agents. Lowest yield loss was recorded indifenconazole (3.84%) followed by propiconazole (9.58%). The remaining treatments recorded relative yield losses more than 12.00%.

Correlation and regression between yield and disease parameter. Correlation and regression analysis were worked-out to know the relationship between yield and disease severity. The results revealed that, highly significant negative correlation exist between yield and per cent disease index (0.965**). The linear correlation between per cent disease index and yield showed negative correlation (R^2 = 0.99) (Fig. 4). Obviously, the yield was decreased with the increase in per cent disease index. Accordingly, regression equation was developed between per cent disease index and yield. A linear negative correlation between yield and PDI was observed representing the best fit having R^2 = 93.5. [Y=219.41-10.62(x)].

Application of bio-control agents like *T. harzianum*, *P. fluorescens* and *B. subtilis* initiates number of biochemical changes, which triggers plant defense responses (Verma and Saharan 1994). Loganathan (2002) reported that induction of defense related proteins *viz.,* phenylalanine ammonia lyase, peroxidase, polyphenol oxidase, phenol, chitinase and β 1-3-glucanase were found to be in higher levels in treatments involving bio-formulation mixture containing *P. fluorescens* against fungal pathogens and root knot nematodes in cabbage and cauliflower. Saikia *et al*. (2004) reported that *P. fluorescens* has different mechanisms to reduce plant diseases such as accumulation of phenolic compounds, increasing activity of PAL, PR-proteins and lysis of the fungal pathogen cell wall by secretion of extra cellular lytic enzymes.

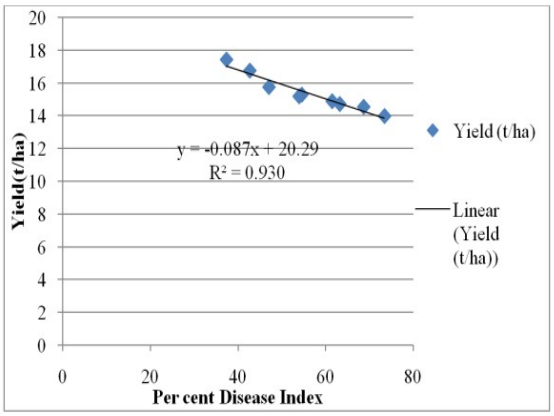

Fig. 4. Correlation between yield and PDI in accordance with different treatments.

Efficacy of these fungicides in controlling black spot and increasing the yields were supported by the findings of Prasad (2014); Chavan *et al*. (2015); Tu *et al.* (2015); Kiran *et al.* (2018); Jackson and Kumar (2019); Meena *et al.* (2020) on cabbage. The effect of *Trichoderma* spp. and bacterial bioagents on black leaf spot on various crucifers in controlling the disease severity and increase in yield were found by Singh *et al.* (2015); Ahmad and Ashraf (2016); Khalse *et al*. (2017); Raghuvanshi *et al.* (2018).

CONCLUSION

Black leaf spot of cabbage caused by different species of *Alternaria* is a foliar fungal disease causing yield loss both qualitatively and quantitatively. The pathogen infecting cabbage was isolated, proved its pathogenicity and identified as *A. brassicicola* based on cultural, morphological and molecular characteristics. The use of fungicides is still the most popular method for management of black leaf spot on crucifers due to no proven source of transferable resistance and is currently unavailable in any of the hosts. In the present study, fungicides belonging to triazole group proved to be effective in reducing the mycelial growth under laboratory conditions and decreased the disease severity by three times by spraying at an interval of 15 days immediately after appearance of disease under field conditions. The field data indicated that for every 1.0% increase in the disease severity, there is 0.9% decrease in the yield with 0.93 confident of R^2.

FUTURE SCOPE

— Development of Integrated package for management of black spot disease of cabbage.
— Identification of source of resistance should be evaluated.

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Conflicts of Interest. None.

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