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Research Article

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In Vitro Evaluation of Botanical Extracts, Chemical Fungicides and *Trichoderma harzianum* Against *Alternaria brassicicola* Causing Leaf Spot of Cabbage

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Abstract

Alternaria leaf spot caused by *Alternaria brassicicola* is one of the destructive diseases of crucifers and causes considerable loss in the yield and qualityof the produce. An experiment was conducted in *in vitro* to evaluate the efficacy of six botanical extracts at three concentrations i.e. 5%, 10% and 15%, six chemical fungicides at five different concentrations i.e. 50ppm, 100ppm, 250ppm, 500ppm and 1000ppm and *Trichoderma harzianum* against *Alternaria brassicicola*.The study was carried out using poisoned food technique for botanical extracts and chemical fungicides and dual culture technique for *T. harzianum* in Completely Randomized Design (CRD). Among botanical extracts, maximum inhibition (99.91%) of mycelial growth was observed in sweet flag at 15% concentration followed by 10% sweet flag (96.68%) and 5% sweet flag (93.64%) and minimum inhibition percent (5.61%) was observed in *Lantana camara* at 5% concentration. Hexaconazole proved to be the most effective chemical fungicide recording 100% growth inhibition at all the tested concentrations which was at par with1000 ppm metalaxyl + mancozeb (Kriloxyl Gold), 500 and 1000 ppm mancozeb and 1000 ppm carbendazim + mancozeb(SAAF)whereas, 50 ppm carbendazim was least effective in reducing fungal growth (7.16%). *T. harzianum* showed 65.02% inhibition of test fungus. The study indicated better performance of some chemical fungicides even at lower concentrations i.e. 100ppm and 250ppm. So, such effective fungicides could be used to minimize hazardous effect. Significant effect of some botanical extracts against pathogen growth suggests their application as potential control agent alternative to chemicals.

Keywords: *Alternaria*, fungicides, plant extracts, poisoned food technique, *Trichoderma*

Introduction

Cabbage (*Brassica oleraceae* var. *capitata* L.) is a leafy head vegetable belonging to Brassicaceae(Cruciferae) family. It is grouped as cole crops, which is originated from a single wild species *Brassica oleracea* var. *oleracea* (*sylvestris*L.), commonly known as wild cabbage or 'Colewort' (Balliu, 2014). Cabbage is native of Western Europe and the northern shore of Mediterranean region (Bose et al. 2001). It grows well in relatively cool, moist climate with moderate to heavy rainfall. It can stand frost in head stage, but freezing temperatures are destructive in other stages. The optimum temperature for seed germination is 12-16 °C and for growth and heading is 15-20 °C, while the growth is arrested above 25°C (Singh, 2007). It is

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biennial crop but grown as annual for vegetable and requires cold treatment for flowering.

Global production of cabbage and other brassicas in 2017 was 71,451,138 mt and the area harvested was 2,513,707 ha (Food and Agriculture Organization Corporate Statistical Database [FAOSTAT], 2019). China ranks first in production (33.42 million mt) followed by India (8.807 million mt) and Russia (3.53 million mt). In Nepal the area cultivated is 29,373 ha and the production is 485,199 mt (Ministry of Agriculture and Livestock Development [MOAD], 2016/17).

Several biotic and abiotic stress affects on its production. Within the biotic stress ,*Alternaria* leaf spot is a common disease incited by several species of *Alternaria.* In cabbage it is caused mainly by two species i.e. *Alternaria brassicae* (Berk.) Sacc., and *A. brassicicola* (Schweintiz) Wiltshire. Symptoms may first develop on young plants in seedbeds, where leaf spots, stunting or damping off may occur. Leaf spots incited by *A. brassicicola* appears as small, dark colored spots which spread rapidly to form circular lesions up to 1 cm in diameter (Singh, 1998). Alternating light and dark concentric rings give the spots the appearance of target; a yellow halo may surround the lesion. The spot incited by large-spore form, *A.brassicae* shows much common symtoms compared to *A. brassicicola* and tend to remain larger and lighter in colour. The fungus is primarily seed-borne, but can also come from crop residue. Spores are spread by wind, water splash, human, agricultural tools and equipments. They can also survive in susceptible weeds or perennial crops (Mamgain et al.,2013). Under *in vitro* conditions sporulation of *A. brassicae* occurs at optimum temperature of 18-24° C and *A. brassicicola* at 20-30° C. Yield losses due to *Alternaria* infection have been reported to be 10-70% in India (Singh et al. 2017; Choudhary et al., 2018) and 32-57% in Nepal (Shrestha et al., 2005; Saharan, et. al., 2016). The disease infect foliage causing extensive damage to tissue involved in photosynthesis and hence result in yield loss.

Conventionally, different fungicides are used for controlling this disease. Indiscriminate use of higher dose of chemical fungicidesaffect environment and human health but also increase input cost (Ahmad & Ashraf, 2016). Several medicinal and aromatic plants show antioxidant and antifungal properties which not only reduce disease development but also produce harmful residue free products (Kavita&Dalbeer, 2015). Use of plant extracts is considered as cost effective and eco-friendly approach of disease management, without any environmental pollution (Khalse et al., 2017). So, the present study was undertaken to screen the effective chemical fungicides and botanical extractswith their optimum concentration and to determine biocontrol potentiality of *Trichoderma harzianum* against *Alternaria brassicicola*.

Materials And Methods

The experiment was carried out in Plant Pathology Laboratory of Institute of Agriculture and Animal Science (IAAS), Lamjung in Completely Randomized Design using poisoned food technique (Nene &Thapliyal, 1979; Thaware,Fugro, Jadhav, Magar&Karande, 2010; Roopa,Yadahalli&Kavyashree, 2014) for botanicals and fungicides screening. Five chemical fungicides i.e. mancozeb, copper oxychloride, hexaconazole, carbendazim + mancozeb and metalaxyl + mancozeb were evaluated at five different concentrations such as 50, 100, 250, 500 and 1000 ppm whereas carbendazim was evaluated at four concentrations i.e. 50, 250, 500 and 1000ppm and six botanical extracts (i.e. neem leaf, garlic bulb, ginger rhizome, *Lantana camara* leaf, sweet flag rhizome and *Artemisia vulgaris* leaf) were evaluated at three different concentrations viz. 5%, 10% and 15% respectively. Biocontrol potentiality of *T. harzianum*was studied using dual culture technique(Babu et al.,2000). Each of the treatment was replicated three times.

Isolation and maintenance of pure culture

Pathogenic *Alternaria brassicicola* was isolated from infected leaf of cabbage collected from field of IAAS, Lamjung. Spores were teased from infected portion for microscopic examination to check the presence of pathogenic fungus. After confirming the presence of *Alternaria brassicicola,* leaves were cut into small pieces (1-1.5cm) with sterile blade. These pieces were disinfected with 0.5% sodium hypochlorite (NaOCl) solution for two minutes followed by three washings with distilled water and excessive moisture was removed using sterile blotting paper. The sterilized leaf pieces were placed on PDA medium using sterilized forceps and incubated at 27 1 °C for 7 days. On the basis of morphological characters of conidia as described by

Yu (2015); Corlett and MacLatchy, (1996a, 1996b) pathogen was identified as *Alternaria brassicicola*. Culture was purified by transferring small piece of agar containing spore to another petri plate containing media and incubated at 27 1 °C for 7 days. The pathogen was sub cultured three times to obtain pure culture and pure culture thus obtained was preserved in PDA slant at 4°C.

In vitro evaluation of botanical extracts and chemical fungicides

Botanical extract was prepared as per methods used by Ul-Haqet al., (2014) and Thaware et al., (2010). Fresh and healthy leaves, bulbs and rhizomes were collected, thoroughly washed in tap water followed by sterilized distilled water, then air dried and grounded with mortar and pestle with the addition of distilled water at the ratio of 1:1 w/v. Then the extract obtained was filtered through double layered muslin cloth. Extract was centrifuged at 4000 rpm for 5 minutes. The supernatant was then filtered through Whatman's filter paper No. 1 and then .boiled at 80° C for 10 minutes in a hot water bath. Thus obtained filtrate was taken as 100% basic stock solution. After autoclaving PDA media and cooling it to 50° C required amount of this standard solution was mixed into PDA to get final concentration of 5%, 10% and 15% for poisoned food technique. Similarly for the evaluation of chemical fungicides, calculated amount of stock solution was mixed in sterilized PDA to make final concentration of 50ppm, 100ppm, 250ppm, 500ppm and 1000ppm. Twenty ml of amended PDA was poured in each 90mm sterilized petri plate and allowed to solidify. Control treatment was maintained without adding plant extracts or chemical fungicides on PDA. A circular disc of 7mm diameter from 9 days old culture of *Alternaria brassicicola* was cut with sterilized cork borer and inoculated in the centre of solidified amended as well as control media. Each treatment was replicated in three petri plates. Then the petriplates were incubated at 27 1 °C for seven days.

Dual culture method

The antagonistic activity of *Trichoderma harzianum*obtained from Nepal Agriculture Research Council (NARC), Khumaltarwas tested against *Alternaria brassicicola* in *in vitro*condition using dual culture technique. Twenty ml of PDA was poured into sterile petri plates. A 7 mm mycelial disc of actively growing *Alternaria*was placed on one end of a petri plate at 1 cm away from the edge. Similarly, 7 mm mycelial disc from 7 days old culture of *T. harzianum* was placed on opposite end of petri plate at1cm away from edge. Plates inoculated with pathogen without biocontrol agent served as a control. Each treatments was replicated in three petri plates. Then the plates were incubated at 27 ± 1 °C for 7 days.

Growth inhibition test

The observation on mycelial growth was recorded after 7 days of incubation in each treatment using vernier caliper scale. The percent growth inhibition of mycelial growth over control was calculated by using the formula given by Vincent (1947, as cited in Kantwa et al., (2014); Roopa et. al, 2014).

$$PGI = \frac{(C-T)}{C} \times 100$$

Where, PGI = Percent growth inhibition, C = Growth of hyphae in control (mm) and

T = Growth of hyphae in treatment (mm)

Statistical analysis

All the data were entered in MS Excel (2013) and analysis of variance was done using RStat software (version 3.5.3). Mean comparison was done using Fisher-LSD test at 0.05 level of significance.

Results And Discussion

In vitro evaluation of botanical extracts

Six different botanical extracts were evaluated at three concentrations for their efficacy against *Alternaria brassicicola* in vitro. The result revealed that, all the tested botanicals inhibited the growth of pathogen over untreated control. Extracts from different plant species used in the experiment showedto possess different level of fungicidal effect against test fungus. Growth inhibition ranged from 32.53% to 96.74% irrespective of concentrations. Significant difference ($P < 0.001$) was obtained among different botanical extracts in their inhibition effect. Increase in effectiveness was observed with increase in concentration. Among three concentrations used, maximum reduction of mycelial growth (74.84%) was observed at 15%

concentrationwhich wassignificantly superior over rest of concentrations. Among the botanical extracts, maximum mean growth inhibition (96.74%) of tested pathogen was recorded in sweet flag followed by neem (77.34%) and garlic (70.05%) while, minimum growth inhibition (32.53%) was recorded in *Lantana camara* followed by *Artemisia vulgaris* (46.77%). In case of interaction effect all concentrations i.e. 5%, 10% and 15% of sweet flag proved to be superior with highest inhibition effect of 93.64%, 96.68% and 99.91% respectively. This was followed by all concentrations (5%, 10% and 15%) of neem (75.87%, 76.00% and 80.15%) and 15% of garlic extract (77.34%) which were statistically at par with each other. *Lantana camara* at 5% concentration showed minimum mycelial growth inhibition of 5.61% followed by *Artemisia vulgaris* at 5% (19.82%), *Lantana camara* at 10% (24.68%) and ginger at 5% (49.72%).

Table 1: *In vitro* efficacy of different botanical extracts on growth of *Alternaria brassicicola.*

S. N.	Treatments	Concentrations						Mean Growth Inhibition (%)
		5%		10%		15%		
		Mean colony diameter (mm)	Growth inhibition (%)	Mean colony diameter (mm)	Growth inhibition (%)	Mean colony diameter (mm)	Growth inhibition (%)	
1	Neem (Azadirachtaindica)	12.84	75.87	12.77	76.00	10.57	80.15	77.34
2	Garlic (Allium sativum)	18.17	65.85	17.58	66.97	12.06	77.34	70.05
3	Ginger (Zingiberofficinale)	26.76	49.72	19.27	63.79	20.66	61.18	58.23
4	Sweet flag (Acoruscalamus)	3.39	93.64	1.77	96.68	0.05	99.91	96.74
5	Wild sage (Lantana camara)	50.23	5.61	40.08	24.68	17.41	67.29	32.53
6	Mugwort (Artemisia vulgaris)	42.67	19.82	22.71	57.31	19.61	63.16	46.77
7	Control	53.22	–--------	53.22	–--------	53.22	–--------	0.00
	Mean CV (%) SEM (±) LSD (p=0.05)		51.75 7.69 2.30 7.08		64.24 4.54 1.68 5.19		74.84 5.49 2.374 7.32	

CV: Coefficient of Variation SEM: Standard Error of Mean LSD: Least Significant Difference

The result was in confirmatory to finding of Pitipong, (2009) who reported complete inhibition of *Alternaria*sp. in *A. calamus*at 1% v/v. Growth of *Alternaria*sp. was completely inhibited above 0.10% concentrations of *A. calamus* (Mungkornasawakul et al., 2002). Sadana&Didwania, (2015) obtained 73.7% inhibition in 15% neem against *A. solani.* Similarly, Kavita and Dalbeer (2015), Waghe et al., (2015), Kakraliya et al., (2018) revealed significant inhibition effect of neem. Chethana et al., (2012), Thaware et al., (2010) and Kantwa et al., (2014) reported better performance of garlic among tested extacts against *Alternaria* sp.The result was in contrary with result of Shrestha and Tiwari, (2009); Biswas and Gosh, (2018). This might be due to inherent physiological differences in different *Alternaria* species. Variation in antifungal activity of different botanicals is due to variation in the content of active antifungal chemicals in the extracts (Shrestha & Tiwari, 2009). Antifungal activity of sweet flag is due to the presence of α- and β-asarone in leaves and rhizomes

(Devi et al., 2014). Scanning electron microscopic study have shown that hyphae and conidia treated with *Acorus* fraction were shrunken and collapsed, which might be due to fluid leakage and some alteration in the membrane permeability (Phongpaichit et al., 2005). Singh et al., (2011) mentioned that, antifungal activity of neem is due to presence of several triterpenoids, peaks and pure compounds (isomeldenin and nimonol))and also Quercetin and β-sitosterol. In addition to these 6-deacetylnimbin, azadiradione, salannin and epoxy-azadiradione are other active compounds. According to Lyer and Williamson, (1991) antifungal properties of neem extracts is attributed to inhibition of protease activity. Inhibition in growth of fungus is due to change in hydrophobicity of cells causing anti-adhesion (Polaquini et al.,2006). Allicin is a key antifungal compound present in garlic and there is presence of phenolics, alkaloids, flavonoids, steroids, glycosides, saponins and tannins (Akinmusire et al.,2014). The effectiveness of garlic clove extract is due to volatile oil which contains dialyldisulphide, diallyltrisulphide and sulphodioxides derived from allicin (Chethana et al.,2012). The allicin or ajoene restricts the performance some enzymes that are important to fungi (Kutawa et al, 2018).

Fungicides

In Vitro Evaluation of Chemical

Table 2: In vitroefficacy of different chemical fungicides on growth of *Alternaria brassicicola*.

S. N.	Treatments	Mean colony diameter (mm) at different concentrations (ppm)					Growth inhibition (%) at different concentrations (ppm)					Mean Growth Inhibition (%)
		50	100	250	500	1000	50	100	250	500	1000	
1	Hexaconazole	0	0	0	0	0	100	100	100	100	100	100
2	Mancozeb	22.51	15.84	0.84	0	0	68.57	77.87	98.83	100	100	89.05
3	Carbendazim	66.46	------	53.28	52.99	44.53	7.16	------	25.58	25.97	37.80	27.84
4	Copper Oxychloride	43.43	35.77	10.91	4.94	4.99	39.33	50.4	84.76	93.10	93.04	72.05
5	Carbendazim + Mancozeb	9.05	6.67	3.89	2.35	0.20	87.36	90.69	94.56	96.72	99.72	93.81
6	Metalaxyl + Mancozeb	18.54	13.53	4.09	3.05	0	74.10	81.10	94.28	95.74	100	89.05
7	Control	71.59					0.00					0.00
	Mean						62.75	79.94	83.00	85.26	88.43	
	CV (%)						6.66	7.07	3.58	2.63	2.26	
	SEM (±)						2.415	3.26	1.715	1.293	1.153	
	LSD (p=0.05)						7.44	10.28	5.29	3.98	3.55	

CV: Coefficient of Variation; SEM: Standard Error of Mean; LSD: Least Significant Difference

The efficacy of different chemical fungicides against test fungus was evaluated *in vitro* using poisoned food technique. The data on inhibition percent is presented in table 2. An insight into data reveals that all the tested chemical fungicides showed significant effect ($P<0.001$) against pathogen growth over control (71.59 mm). The extent of mycelial growth inhibition increased with increase in their concentration. Among the chemicals tested hexaconazole proved to be the most effective fungicide showing complete inhibition (100%) followed by carbendazim+ mancozeb (93.81%) and mancozeb (89.05%) while carbendazim (27.83%) followed by copper oxychloride (72.05%) were found to be least effective. In case of interaction effect, highest inhibition (i.e. 100%) of mycelial growth was recorded in all concentrations of hexaconazole, 1000 ppm of mancozeb and metalaxyl + mancozeb, 500 ppm of mancozeb which were significantly indifferent ($P<0.05$) with 1000 ppm carbendazim + mancozeb (99.71%), 500 ppm of carbendazim + mancozeb (96.72%) and metalaxyl + mancozeb (95.74%) and 250 ppm of mancozeb (98.83%), carbendazim + mancozeb

(94.56%) and metalaxyl + mancozeb (94.58%). Minimum growth inhibition (7.16%, 25.58%, 25.97% and 37.80%) were obtained in all tested concentrations (50, 250, 500 and 1000 ppm) of carbendazim followed by 50 ppm copper oxychloride (39.33%).

Similar result was recorded in findings of Panwar et al., (2013) who reported complete growth inhibition of *A. alternata* in hexaconazole followed by mancozeb and least inhibition in carbendazim. Similarly, Tu (2015) recorded complete inhibition of *A. brassicicola* by hexaconazole, mancozeb at 250, 500 and 1000 ppm and by metalaxyl + mancozeb at 500 and 1000 ppm and least inhibition at carbendazim. Roopa,Yadahalli and Kavyashree (2014) found mancozeb as a superior contact fungicide, hexaconazole as a superior systemic fungicide and carbendazim +mancozeb as superior combination fungicide against *A. solani.* Biswas and Ghosh (2018), Kantwa et al., (2014) obtained significant growth inhibition effect of *Alternaria* sp. in mancozeb. Similar inhibition was observed by Thawareet al., (2010) against *A. alternata.*Gautam et al., (2018) reported 93.65%, 87.65% and 76.7% growth inhibition of *A. brassicae* at 0.1, 0.01 and 0.001% concentrations of SAAF (carbendazim + mancozeb) respectively. SAAF effectively controlled the *Alternaria* blight of sunflower in field experiment of Wagheet al., (2015). Synthetic fungicides bring about the inhibition of pathogens either by destroying theircell membrane or its permeability or by inhibiting metabolic processes of the pathogen and hence are effective (Kakraliya et al., 2018). Higher inhibition effect of hexaconazole is due to inhibition of ergosterol biosynthesis, controlling the growth and reproduction of fungal pathogen (Muhamad et al., 2010). Mancozeb being multisite inhibitor effects lipid metabolism, respiration and production of ATP, it interferes with enzymes containing sulphydryl groups, disrupting different biochemical process within the fungal cell cytoplasm and mitochondria.

In Vitro evaluation of *Trichoderma harzianum* against *Alternaria brassicicola*.

In this study, the antagonistic effect of *T. harzianum* was assessed against *Alternaia brassicicola* using dual culture technique. Growth inhibition by 65.02% of *Alternaria spp*. was obtained after seven days of incubation as compared to control. The result is in agreement with Ganie et al., (2013), who reported 71.85% growth inhibition of *A. solani.* Such inhibitory effect of *T. harzianum* have been recorded by Wagheet al., (2015) and Kulkarni et al., (2014). The antagonistic activity of *Trichoderma* is mainly due to production of acetaldehyde compound (Kithan&Daiho, 2014). Nutrients and niche competitions, antibiosis by production of volatile components and non-volatile antibiotics could be possible cause of antagonism (Hajieghrari et al., 2008; Kumar et al., 2011). Inhibitory activity of *Trichoderma spp*. may be due to secretion of extracellular cell degrading enzymes such as chitinase, β-1,3-glucanase, cellulose, lectin (Kakraliyaet al., 2018) and other secondary metabolites such as glioviridin, viridian and gliotoxin which may help mycoparasites in colonization of host (Patel, Lal and Singh, 2014).

Conclusion

Alternaria leaf spot is a worldwide disease of economic importance in brassica vegetables. Different chemical fungicides are commercially available in market to control this disease. This study revealed significant inhibition effect of all the tested chemical fungicides and botanical extracts over control. Indiscriminate application of chemical fungicides have resulted several health hazards, negative impacts in environment so, the use of effective chemical at possible lower concentrations could be safer way to minimize health hazards and environmental pollutions. *T. harzianum* and botanical extracts such as sweet flag, neem and garlic exhibited inhibition of *Alternaria brassicicola* at higher percentage. Therefore, this biological agent and plant extracts could be a potential to be used as novel fungicides alternative to harmful chemical fungicides. However, these *in vitro* research finding should be verified in the field conditions before taking for field application.

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