In-vitro Evaluation of Botanicals, Fungi-toxic Chemicals and Bio-control Agent for Efficacy against Turcicum Leaf Blight of Maize

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ABSTRACT

Maize is the second most important cereal crop of Nepal. Its growth and production is severely affected by Turcicum leaf blight caused by Exserohilum turcicum at pre-harvest stage. A total of 6 botanicals, 4 chemical fungicides and 1 bio-control agent were evaluated for efficacy against Exserohilum turcicum under in vitro conditions following poisoned food technique at National Maize Research Program, Rampur, Nepal. The experiment was carried out in a completely randomized design with 5 replications. All the tested botanicals, fungicides and bio-control agent exhibited fungicidal action and significantly inhibited mycelial growth of the test pathogen over untreated control. Among botanicals, extract of Acorus calamus L. at 1% W/V checked the pathogen growth completely in-vitro. The mycelial growth inhibition percent of Artimisia indica Willd, Lantana camera L., Allium sativum L., Xanthoxylum armatum DC., and Azadirachta indica A. Juss. at the concentration of 2.5% W/V on PDA was 75.18%, 74.00%, 44.68%, 44.21% and 37.59% respectively. Among fungicides, the mycelial growth inhibition percent of E. turcicum due to ACME-COP (Copper oxychloride 50% WP), SAAF (Carbendazim 12% + Mancozeb 63% WP), Dithane M-45 (Mancozeb 75% WP) and Bavistin (Carbendazim 50% WP) at the concentration of 1000 ppm on PDA was 70.69%, 68.44%, 61.23%, and 60.52% respectively. Antagonist Trichoderma viride developed more rapidly than E. turcium in single as well as in dual cultures. T. viride caused significantly inhibition of 35% of the pathogen E. turcicum on the 5th day of incubation. These results have important implications for the management of turcicum leaf blight disease in maize.

Keywords: Biological control, Exserohilum turcicum, fungicides, mycelial inhibition, plant extracts.
INTRODUCTION
There are various biotic and abiotic stresses which are responsible for the feeble yield of maize (Zea mays L.) in Nepal; however, different diseases and poor crop management practices are most important among them (Subedi 2015). Maize is invaded by several fungal pathogens, but ubiquitous incidence of turcicum leaf blight caused by Exserohilum turcicum at pre-harvest stage is the most important bottleneck in satisfactory maize production (NMRP 2015). Heavy dews, cool temperature and frequent rains are environmental conditions conducive for disease development (Jordan et al. 1983). The pathogen first identified in Nepal in 1964 from Patan, Lalitpur (Khadka and Shah 1967, Manandhar 1980). The disease is widely distributed throughout the country; however, it is more prevalent in the cool and humid conditions of the foothills and also common in the winter maize of the terai region. It was not considered as the major disease of maize crop in Nepal until 1985 (Dahal et al. 1992). The disease usually appears before tasseling cause yield loss up to 40 to 70% and has become the most important disease at present in Nepal (NMRP 2016). Late sown maize in the summer season is most affected by this disease in hills (Manandhar et al. 2007). The objective of this work was to evaluate the plant extracts, chemical fungicides and bio-control agents for their efficacy against Exserohilum turcicum causing northern leaf blight of maize under in vitro condition.

MATERIALS AND METHODS

Experimental site and isolation of pathogen
The samples of northern leaf blight disease specimens were collected from maize (Zea mays L. 2n = 2x = 20) growing maize fields of National Maize Research Program (NMRP), Rampur and brought in to the Plant pathology laboratory during winter season of 2017. The infected leaves were cut into several small sections 5-10 mm-square from the margin of the infected lesion to contain both diseased and healthy tissue. Then they were placed in surface sterilizing agents’ solutions (1% NaOCl) for about 15-30 seconds and the sections were taken out aseptically and blotted dry on clean, sterile paper towels or washed in three changes of sterile water and placed on the nutrient medium, usually three to five sections per dish. The pathogen, E. turcicum, grew from the sections and the colonies of the pathogen were sub cultured aseptically for further study.

Botanicals and preparation of extracts
The botanicals selected for the experiments were Sweet flag (Acorus calamus L. 2n = 4x = 48), Prickly ash (Xanthoxylum armatum DC. 2n = 2x = 66), Mugwort (Artimisia indica Willd. 2n = 2x = 18), Neem (Azadirachta indica A. Juss. 2n = 2x = 28), Lantana (Lantana camera L. 2n = 2x = 22, 44) and Garlic (Allium sativum L. 2n = 2x = 16). The dried fruits of prickly ash, stolos of sweet flag, garlic and fresh leaves of neem, mugwort and Lantana camera, were used to prepare the extract. These materials were dried in the shade for two days. While preparing the extracts, the unwanted debris was removed, surface sterilized for 2 minutes in 70% ethanol, and washed in three changes of sterile distilled water. The paste was obtained by grinding 1, 2 and 2.5g each of prickly ash, sweet flag, neem, garlic, mugwort and lantana plants separately in 100 ml of sterile distilled water using a mortar and pestle. The paste was squeezed and filtered through 4 folds of sterile cotton wool into a 150 ml conical flask to give concentrations of 1, 2 and 2.5% W/V (Wokocha and Okereke 2005). The volume of the filtrate collected was 100 ml and each of the extract concentration was kept aseptically in 150 ml conical flasks. The content in the flasks was exposed to UV light for further sterilization.

Fungicides and bio-control agents
The chemical fungicides used for the experiment were Dithane M-45 (Mancozeb 75% WP), SAAF (Carbendazim 12% + Mancozeb 63% WP), ACME-COP (Copper oxychloride 50% WP) and Bavistin (Carbendazim 50% WP). The concentration level maintained was 1000 ppm. The bio-control agent used for the experiment was laboratory isolated culture of Trichoderma viride.

In-vitro test
The in-vitro test of plant extracts and fungicides against Exserohilum turcicum was done at the laboratory of NMRP, Rampur, Chitwan during 2017 winter. The experiment was carried out by poisoned food technique with completely randomized design (CRD). The linear growth was measured after 15 days of incubation. Different concentrations of water extracts of plants and fungicides (1000
ppm) were individually incorporated into different conical flasks containing 100 ml of sterile molten potato dextrose agar medium prepared from the dehydrated PDA (HIMEDIA, REF MO96-500G) as per the instructions of the manufacturer in the laboratory condition. The plant extracts and fungicides were added to the PDA separately when it was cooled to about 50°C with the help of micro-pipette. The flasks were gently agitated for 2 min to allow for a proper mixing of extract with the nutritive medium then 20 ml medium was poured in the petri-plates (9cm diameter). Strepto-penicillin (Bistrepen-V) (250 mg per litre) was added to the medium at the time of pouring to prevent bacterial contamination. In case of plant extracts, there were a total of seven different treatments consisting of prickly ash, sweet flag, mugwort, neem, lantana, garlic and control. The concentration of botanicals was maintained to 1, 2 and 2.5% W/V. There were 6 experimental setting consisting of prickly ash, sweet flag, mug-wort, neem, lantana and garlic extract. Each experiment included 4 treatments; plant extract at 1% W/V, 2% W/V and 2.5% W/V including control (without any extract). In case of fungicides, there were 5 treatments consisting of Dithane M-45 (Mancozeb 75% WP), SAAF (Carbendazim 12% + Mancozeb 63% WP), ACME-COP (Copper oxychloride 50% WP), Bavistin (Carbendazim 50% WP) and control. The concentration level maintained for the fungicides was 1000 ppm. Four mm diameter of *E. turcicum* of one week old culture was cut by cork borer and picked up with the help of inoculating needle and placed onto the center of the plate. The plate contained PDA amended plant extract and fungicides where the cut piece was kept upside down for better contact of pathogen to the media. The plates were incubated in incubator at 25°C for up to 15 days.

**Observation**

The colony diameter (mm) of the pathogen was determined by measuring the average radial growth on different incubation dates. Average radial growth was recorded by using a measuring scale from the lower view of the petri-plates.

Mycelial growth inhibition percent was calculated by following formula.

Mycelial growth inhibition (%) = \[(dc-dt) / dc\] × 100

Where dc = average diameter of fungal colony in the control and dt = average diameter of fungal colony in the treatment group

The antagonist effect of bio-control agent was evaluated against *E. turcicum* in dual culture technique. Four mm diameter of tested pathogen of one week old culture and above stated bio control agent was cut by cork borer and picked up with the help of inoculating needle and placed apart on solidified PDA medium in an equal distance comprising 5 replications and incubated as stated earlier. Observations were recorded by measuring colony diameter of both the test fungus and bio agent on different days of incubation up to 5 days and per cent inhibition was calculated.

**Statistical analysis**

The recorded observations were analyzed statistically using MSTAT-C computer package program and treatment means were compared using Duncan’s Multiple Range Test (DMRT) at 1% levels of significance

**RESULTS**

All tested botanicals inhibited radial mycelial growth of *Exserohilum turcicum* significantly over the control at different concentrations (Table 1). The sweet flag (*Acorus calamus* L.) even at lower dose (1% W/V on PDA) was able to check completely the growth of pathogen (4.00 mm). With the increase in concentration (2.5% W/V), the mean colony diameter of the *E. turcicum* in the plate amended with mugwort (*Artimisia indica* Willd) and lantana (*Lantana camera* L.) was significantly lower i.e. 21.0 mm and 22.0 mm respectively than the diameter recorded at control plate. However, the mean colony diameter of the pathogen did not differ significantly at 2.5% W/V dose when the plates were amended with prickly ash (*Xanthoxylum armatum* DC.) and garlic (*Allium sativum* L.) separately up to 15th day of incubation period (Figure 1). The mycelial growth inhibition percent of *Artimisia indica* Willd, *Lantana camera* L., *Allium sativum* L., *Xanthoxylum armatum* DC., and *Azadirachta indica* A. Juss. at the concentration of 2.5% W/V on PDA was 75.18%, 74.00%, 44.68%, 44.21% and 37.59% respectively (Table 2).
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Table 1. Effect of plant extracts incorporated PDA on the growth of E. turcicum in different concentration levels at 25°C temperature during 2017 at NMRP, Rampur, Chitwan, Nepal

| Treatment          | Mean colony diameter (mm) | 1% W/V | 2% W/V | 2.5% W/V |
|--------------------|---------------------------|--------|--------|----------|
| Acorus calamus     |                           | 4.00   | 4.00   | 4.00     |
| Xanthoxylum armatum| 73.00 c                   | 57.00 c| 47.20 c|
| Artimisia indica   | 43.60 f                   | 23.80 e| 21.00 d|
| Azadirachta indica | 56.00 d                   | 55.40 c| 52.80 b|
| Lantana camera     | 57.60 d                   | 26.80 d| 22.00 d|
| Allium sativum     | 79.00 b                   | 72.80 b| 46.80 c|
| PDA only           |                           | 84.60 a| 84.60 a| 84.60 a  |
| F test             | **                        | **     | **     |          |
| LSD (0.01)         |                           | 2.42   | 2.66   | 3.13     |
| CV%                |                           | 2.43   | 3.28   | 4.50     |

Means of 5 replications. Means in column with same superscript are not significantly different by LSD (P<0.01). mm-millimeter, W/V-Weight by volume, PDA = Potato Dextrose Agar, **- highly significant.

Table 2. Effect of plant extracts incorporated PDA on mycelial growth inhibition of E. turcicum in different concentration levels at 25°C temperature during 2017 at NMRP, Rampur, Chitwan, Nepal

| Treatment          | MGI %       | 1% W/V | 2% W/V | 2.5% W/V |
|--------------------|-------------|--------|--------|----------|
| Acorus calamus     | 95.27       | 95.27  | 95.27  |
| Xanthoxylum armatum| 13.71       | 32.62  | 44.21  |
| Artimisia indica   | 48.46       | 71.87  | 75.18  |
| Azadirachta indica | 33.81       | 34.52  | 37.59  |
| Lantana camera     | 31.91       | 68.32  | 74.00  |
| Allium sativum     | 6.62        | 13.95  | 44.68  |
| PDA only           |             |        |        |          |
| MGI- Mycelial Growth Inhibition, W/V-Weight by volume, PDA=Potato Dextrose Agar |

All the tested fungicides incorporated PDA had significant (p<0.01) effect on the growth of Exserohilum turcicum at 25°C as compared to control at 1000 ppm concentrations up to 15th day of incubation (Table 3). There was no significant difference between Bavistin (Carbendazim 50% WP) and Dithane M-45 (Mancozeb 75% WP) to reduce the growth of Exserohilum turcicum in all the dates of incubation. The fungicides SAAF (Carbendazim 12% + Mancozeb 63% WP) and ACME-COP (Copper oxychloride 50% WP) had also the same effect to check the growth of pathogen (Figure 2). The mean colony diameter of the pathogen in the plate amended with ACME-COP (Copper oxychloride 50% WP), SAAF (Carbendazim 12% + Mancozeb 63% WP), Dithane M-45 (Mancozeb 75% WP) and Bavistin (Carbendazim 50% WP) was significantly lower ie 24.80 mm, 26.70 mm, 32.80 mm and 33.40 mm respectively than the control plate (84.60 mm) on 15th day of incubation. The mycelial growth inhibition percent of E. turcicum due to ACME-COP (Copper oxychloride 50% WP), SAAF (Carbendazim 12% + Mancozeb 63% WP), Dithane M-45 (Mancozeb 75% WP) and Bavistin (Carbendazim 50% WP) at the concentration of 1000 ppm on PDA was 70.69%, 68.44%, 61.23%, and 60.52% respectively.

Table 3. Effect of fungicides incorporated PDA on the growth of E. turcicum in concentration level (1000 ppm) at 25°C during 2017 at NMRP, Rampur, Chitwan, Nepal

| Treatments (Concentration: 1000 ppm) | Mean colony diameter (mm) | MGI% |
|-------------------------------------|---------------------------|------|
| Dithane M-45 (Mancozeb 75% WP)      | 32.80                   | 61.23|
| SAAF (Carbendazim 12% + Mancozeb 63% WP) | 26.70                  | 68.44|
| ACME-COP (Copper oxychloride 50% WP)| 24.80                   | 70.69|
| Bavistin (Carbendazim 50% WP)       | 33.40                   | 60.52|
| Control (only PDA)                  | 84.60                   |      |
| F test                              | **                      |      |
| LSD (0.01)                          | 3.15                    |      |
| CV%                                 | 4.33                    |      |

Means of 5 replications. Means in column with same superscript are not significantly different by LSD (P<0.01) mm-millimeter, MGI-Mycelial Growth Inhibition, ppm-parts per million, WP-wettable powder, PDA = Potato Dextrose Agar, **- highly significant.
Figure 1. Effect of plant extracts on the growth of *E. turcicum* in different concentration levels at 25°C during 2017 at NMRP, Rampur, Chitwan

Figure 2. Effect of fungicides on the growth of *E. turcicum* in different concentration levels at 25°C during 2017 at NMRP, Rampur, Chitwan

The mean colony diameter of the antagonist was significantly higher than the pathogen, *E. turcicum*, on 5th day of incubation at 25°C (Table 4). Less (18.60 mm) mean colony diameter of the pathogen was measured when dual culture was done with *Trichoderma viride* Pers. (PPD isolate). The diameter of the pathogen alone was 28.60 mm on 5th day of incubation at room temperature. The mean colony diameter of the antagonist *T. viride* was noticed higher in dual culture with *E. turcicum* in all days of incubation compared to the diameter of *T. viride* grown alone in PDA (Figure 3). It was observed that the growth of the antagonist was enhanced in dual culture with pathogen than the antagonist alone grown in PDA while the growth of the pathogen was less in dual culture with antagonist compared to the pathogen grown alone in PDA in all days of incubation.

**Table 4. Effect of *Trichoderma viride* on the growth of *E. turcicum* in different incubation periods at 25°C during 2017 at NMRP, Rampur, Chitwan**

| Treatment                                      | 1st day | 2nd day | 3rd day | 4th day | 5th day |
|-----------------------------------------------|---------|---------|---------|---------|---------|
| *Trichoderma viride* only                     | 20.80a  | 50.20b  | 68.00b  | 83.20a  | 88.80a  |
| *T. viride* in dual culture with *E. turcicum*| 28.80a  | 61.80a  | 73.90a  | 85.10a  | 88.30a  |
| *Exserohilum turcicum* only                   | 11.00c  | 14.40c  | 21.80c  | 24.40c  | 28.60c  |
| *E. turcicum* in dual culture with *T. viride*| 7.40d   | 13.60c  | 15.40d  | 17.80c  | 18.60c  |
| F test                                        | **     | **      | **      | **      | **      |
| LSD (0.01)                                    | 2.00    | 2.63    | 3.67    | 2.92    | 2.69    |
| CV%                                           | 6.38    | 4.07    | 4.44    | 3.00    | 2.60    |

<sup>1</sup>Means of 5 replications. Means in column with same superscript are not significantly different by LSD<sub>(P<0.01)</sub> mm-millimeter. **-, highly significant
**DISCUSSION**

As the plant pathogens develop resistance to many fungicides it creates immense problem to control various crop diseases which pushes to search for new and alternative fungi-toxic substance from various sources including plant extracts. The results indicated that the various phyto-extracts tested inhibited the mycelia growth of *E. turcicum* as compared to control. However, there was no further growth of the pathogen on the PDA incorporated with *Acorus calamus* extract at 1% W/V in all the dates of incubation. Even at the lower dose 0.5 % W/V on PDA, *Acorus calamus* extract significantly check the growth of the pathogen followed by *Artimisia indica* and *Lantana camera* at higher concentration level (2.5% W/V). Similar results on antifungal activity of aqueous extracts of different plants has been reported by various workers (Shivapuri et al 1997, Meena et al 2003). From the present study, it is concluded that *Acorus calamus* L., *Artimisia indica* and *Lantana camera* L. were effective botanicals to suppress the growth of *E. turcicum*. A perusal of literature showed similar experiments regarding the control of *E. turcicum* using plant extracts has not yet been done in Nepal and very few literatures were available. Phongpaichit et al(2005) investigated that an antimicrobial guided one of methanol extracts of *A. calamus* FRQWDLQLQJ-asarone as a major component showing high antifungal activity against *Microsporum gypseum* Bodin, *Trichophyton rubrum* Castell. and *Penicillium marneffei* Segretain and had moderate activity against *Candida albicans* Berkhout and *Cryptococcus neoformans* Vuill. The findings of Kungha (1999) also indicated that alcoholic extract of *A. calamus* could inhibit many fungi including *Alternaria brassicaceae* Sacc, *Fusarium oxysporum* f.sp. lycopersici, *Rhizoctonia solani* Kuhn, *Sclerotiana sclerotiorum* de Bary at the concentration of 0.10% upward. The major antifungal compound found in *A. calamus* is β-asarone which is considered to be the most biological active compound in the rhizome of *A. calamus* and quantities of β-asarone vary 10-20% in the rhizome of European origin while in Asia, it was found in the range of 70-90% (Karwowska et al 1997). Findings of current study are in agreement with the report of Deena and Thoppil (2000) who described the essential oil of *Lantana camera* containing β-caryophyllene, geranyl acetate, terpinyl acetate,bornyl acetate and limonene remarkably inhibited the growth of many tested bacteria and fungi. *Aspergillus niger* Tieghem, *Fusarium solani* Schlecht and *Candida albicans* Berkhout appeared as the most sensitive ones. Khedkar et al (2012) studied the efficacy of phyto extract on *E. turcicum* by poisoned food technique under in vitro conditions and found that Nimbicidin @ 5% concentration was superior to all other treatments with the maximum mycelial inhibition of 68.85% followed by negunda (38.88%) and neem seed kernel extract (37.01%). Subedi et al (2015) also reported that *Acorus calamus* L. was the most effective botanical followed by *Xanthoxylum armatum* when tested against *Stemphylium botryosum* Walr. both at in vitro and in vivo condition.

The finding of this experiment is also in agreement with the Wani et al (2017) who found that among fungicides, Mancozeb was found best (95.23% mean inhibition) and among plant extracts, neem oil inhibited maximum mycelial growth of *E. turcicum* (62.33%). Investigations also indicated that
mancozeb was most effective in controlling Turcicum leaf blight of maize (Pandurangegowda 1987, Begum et al 1993). Reddy et al (2013) evaluated seven fungicides in vitro against *E. turcicum* causing leaf blight of maize and found treatment mancozeb @ 0.25% and combination treatment of mancozeb and carbendazim, i.e, Saff @ 0.25% recorded the lowest per cent disease index (PDI) reducing the disease by 73.0 and 72.1%, respectively.

Antagonist *Trichoderma viride* Pers. showed the potentials to suppress the radial colony growth of *E. turcicum* under laboratory condition. *T. viride* developed more rapidly than *E. turcicum* in single as well as in dual cultures. The intensive development of *Trichoderma* gives it a significant advantage in competition with pathogens for nutrients and space, besides the production of mycotoxins. *In vitro* evaluation of bio-control agents revealed that *Trichoderma viride* caused significantly inhibition (35%) of the pathogen *E. turcicum* on 5th day of incubation. The inhibitory effect of these bio-agents was probably due to competition and / or antibiosis. The antagonism of *T. viride* observed in the present studies is in tune with the findings of various workers (Biles and Hill 1988, Mahamood et al 1995, Ramachandra 2000). The mechanism of *Trichoderma* to control pathogens may be by attacking and binding the pathogenic organisms by sugar linkage and begins to secrete extracellular protease and lipase (Cal et al 2004). *Trichoderma sp.* grown over the pathogenic fungal hyphae, coils around them and degrades the cell walls. This action of parasitism restricts the development and activity of pathogenic fungi. Additionally, or together with mycoparasitism, some *Trichoderma* species release antibiotics (Harman 2006). According to Rosado et al (2007), the main factor for ecological success of this genus is a combination of very active mycoparasitism mechanisms and an effective defensive strategy, induced in the plants.

All the tested botanicals, fungicides and bio-control agent exhibit fungicidal action and inhibit mycelial growth of the *Exserohilum turcicum*. Among botanicals, extract of *Acorus calamus* L. at lower dose also completely check the pathogen growth *in-vitro*. At higher dose, plant extracts of *Artimisia indica* Willd, *Lantana camera* L., *Xanthoxylum armatum* DC., *Allium sativum* L. and *Azadirachta indica* A. Juss. are also effective to reduce the mycelial growth of *E. turcicum*. Among fungicides, ACME-COP (Copper oxychloride 50% WP), SAAF (Carbendazim 12% + Mancozeb 63% WP), Dithane M-45 (Mancozeb 75% WP) and Bavistin (Carbendazim 50% WP) at the concentration of 1000 ppm on PDA are effective to check the growth of the pathogen. Antagonist *Trichoderma viride* Pers. suppresses the radial colony growth of *E. turcicum* and develops more rapidly than *E. turcicum* in single as well as in dual cultures.

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