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In vitro antagonistic efficacy of plant extracts on the enzyme activity of *Colletotrichum gloeosporioides*

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Anthracnose fungus, *Colletotrichum gloeosporioides* was isolated in culture from the diseased fruit samples of chilli (*Capsicum annuum*). The fungus was identified by morphological and molecular analysis. Plant root extracts of *A. precatorius* and *R. tetraphylla* and chemical fungicide, dithane-M45 were tested for their inhibitory effects on the cell wall degrading enzymes of *C. gloeosporioides* under *in vitro* condition. The activity of cell wall degrading enzymes viz. polymethyl galacturonase (PMG), pectin transeliminase (PTE) and the carboxymethyl cellulase (CMC) was assayed. Root extracts of *A. precatorius* was found to be more efficient in inhibiting both the pectinolytic (PMG and PTE) and cellulolytic (CMC) enzymes than dithane-M45. The inhibitory effects of enzyme might be due to the presence of fungitoxic compounds such as phenolics, proteins and flavonoids in the root extracts of *A. precatorius*. On the contrary, extract of *R. tetraphylla* was found to induce enzyme activity.

Key words: Plant extract, botanical pesticide, pectinolytic enzyme, cellulolytic enzyme.

INTRODUCTION

Chilli (*Capsicum annuum* L.) is infected by different fungal pathogens, among them anthracnose is the most serious fungal disease and is caused by *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc. (Than et al., 2008; Narasimhan and Shivakumar, 2012; Suwan et al., 2012). *Colletotrichum gloeosporioides* has also been reported to cause anthracnose in many tropical fruit trees such as mango, papaya (Bussaman et al., 2012; Awa et al., 2012; Choi et al., 2012; Pandey et al., 2011). This disease is very harmful and can cause spoilage and rotting of fruit plants, resulting in low yield and poor quality of the fruits (Peraza-S´anchez et al., 2005). It is estimated that the yield loss due to *C. gloeosporioides* infection in chilli was 25%. Degradation of cell wall by pathogen in soft roots is one of the important steps in symptom development. One of the most conspicuous effects of microorganisms on plant cell wall is enzymatic degradation (Walton, 1994) of cell wall degrading enzymes produced by *Colletotrichum* spp. has already been reported by many researchers (Anand et al., 2008; Wattod et al., 1994; Chacko et al., 1995). Cell wall degrading enzymes are one of the basic compatibility factors, required for penetration and ramification and for obtaining nutrients from wall polymers (Walton, 1994; Yakoby et al., 2000; Mwenje and Mguni, 2001). Antimicrobials and antifungal compounds of plant origin are much preferred to synthe-

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tic compounds in vogue, since they are environmentally safe, easily degradable and leave no harmful and hazardous residues (Bussaman et al., 2012; Johnny et al., 2011;Hernandez-Albiter et al., 2007; Ivy and Sekhar, 1997; Fawcett and Spencer, 1970; Imtiaj et al., 2005). Gangawane (1990) reported that *Colletotrichum* sp. developed resistance against the commercially available fungicides. Though much work has been carried out in exploring the fungicides of botanical origin, there has been no detailed study on the effects of plant root extracts on the cell wall degrading enzymes of pathogenic fungi. Earlier**,** *in vitro* studies on the effect of ethanolic root extract of *A. precatorius* and *Rauvolfia tetraphylla* showed significant inhibition on the conidial germination and mycelia growth of *Colletotrichum* sp. (Kumaran et al., 2003). The present study reports the efficacy of ethanolic root extracts of *A. precatorius* and *R. tetraphylla* and dithane-M45 on the activity of cellulase (carboxymethyl cellulase – CMC) and pectinases (polymethyl galacturonase – PMG and pectin trans eliminase – PTE) enzymes in *in vitro* condition.

MATERIALS AND METHODS

Isolation, identification and maintenance of the fungus

Colletotrichum gloeosporioides was isolated from the infected fruit tissues of Chilli (*Capsicum annuum*). Infected fruit were cut into small sections and surface-sterilized in 0.1% mercuric chloride for 60s and then rinsed thrice in sterile distilled water. The infected sections were inoculated on potato dextrose agar (PDA) medium amended with streptomycin (to prevent bacterial growth) incubated at 26 ± 2°C. The hyphal tips, which grew out from the plant segments were isolated and sub-cultured onto PDA and brought into pure culture. Photomicrograph of conidia was taken using Nikon Optiphot trinocular microscope (bright field) with Nikon Coolpix900 digital camera. The isolated fungus was identified by their morphological and molecular analysis. The analysis of 5.8S and 28S ribosomal RNA sequence of the isolates was achieved using polymerase chain reaction (PCR). DNA extraction, PCR amplification and sequencing were carried out using standard methods (Kumaran et al., 2011; Weir et al., 2012). The rRNA sequences of the test fungus were compared with the NCBI site database. Nucleotide BLAST program of gene bank were used to estimate the percentage of similarity/homology among the available fungal species. The test fungus (no.MEKU-27) was deposited at the University laboratory culture collection.

Preparation of root extracts

Ten percent (w/v) concentration of plant extracts were used in this study. The primary and secondary fresh roots of *A. precatorius* and *R. tetraphylla* were surface sterilized with 0.1% mercuric chloride solution. The roots were chopped and softened by using a sterile wooden mortar and pestle. They were soaked in 80% ethanol for seven days. The ethanolic extracts were then evaporated in a desicator with KOH pellets under vacuum. The dry extract was dissolved in sterile distilled water with equal weight by volume of the root and centrifuged for 10 min at 5000 rpm. The supernatant was collected and stored at 4°C. The root extracts were added to broth and final concentration in the culture medium was made to 10%. Concurrently chemical fungicide dithane-M45 at 320 ppm concentration was also tested (Josef, 1984; Bhuiyan et al., 2012).

Enzyme production

To 50 ml Czapek's Dox broth containing 10% concentration root extracts, two mycelia discs of *C. gloeosporioides* (9mm diameter) were inoculated and incubated at 26 ± 2°C for 7 days. Culture medium devoid of plant extract served as the control. Pectinolytic and cellulolytic enzymes were produced by adding 2% pectin and 1% carboxy methyl cellulose to the growth media respectively as the carbon source. Culture were filtered with Whatman No.1 filter paper and centrifuged (5000 rpm/10 min) in a high speed-cooling centrifuge. Enzymes were stored at 2-4°C under a thin layer of toluene to avoid contamination and to be used within a week. The culture filtrates were then used for viscometric and calorimetric assays.

Enzyme activity assay

PMG (Enzyme Commission (EC) number, EC 3.2.1.67), PTE (EC 4.2.2.10) and CMC – endoglucanase (EC 3.3.1.4) activities were assayed by estimating the loss of viscosity of substrate by viscometric method using Ostwald Viscometer-150 (Mahadevan and Sridhaar, 1986; Aboaba, 2009; Akintobi et al., 2012). Suction was first fixed to the large arm of the viscometer to mix the contents and then to the small arm to determine the viscosity of the reaction mixture (that is in zero time). The efflux time of the mixture at 28°C was measured for 3h at 30 min interval and the percentage of loss in viscosity was calculated using the formula,

$$V = T_o\text{-}T/T_o\text{-}T_w \times 100$$

Where, V is the percent loss in viscosity; T_o is the flow time of reaction mixture at zero time; T is the flow time of the reaction mixture at a particular time interval and T_w is the flow time of distilled water.

In all the cases the enzyme activity was expressed as relative activity (RA) units. One RA is equal to reciprocal of time taken for 25% viscosity loss in minutes X 10^3.

For PMG, the enzyme reaction mixture contained 4ml of 1% pectin in acetate buffer pH 5.2, 2 ml of the culture filtrate and 1ml acetate buffer at pH 5.2.

Viscometric assay for PTE was carried out similar to PMG, but buffer used was boric acid and borax buffer at pH 8.7. The exo activity of enzyme was also assayed by calculating the amount of thiobarbituric acid (TBA) reacting substances produced in the culture filtrate (Mahadevan and Sridhar, 1986; Aboaba, 2009). Three mililiter of the reaction mixture was drawn after 3 h of incubition and 10 ml of 0.01M TBA and 5 ml of 0.5N HCl was added to stop the reaction. The reaction mixture was placed in a boiling water bath for 60 min, cooled under running tap water, centrifuged at 5000 rpm for 15 min and then the supernatant was collected. Absorbance was read at 547 nm. Enzyme-substrate mixture drawn at zero time was used as the control. The activity was expressed as specific activity (SA) units and one SA is equal to the changes in absorbance of 0.001 at 547nm/h/ml enzyme.

For β-1,4 endoglucanase, viscometric assay of the reaction mixture contained 2ml of culture filtrate and 4 ml of 0.5% carboxy methyl cellulose substrate dissolved in 1ml of acetate buffer at pH 5.2. In addition to viscometric method, exoactivity of endo-1,4-β glucanase activity was determined by the amount of reducing sugars liberated in a 3h incubated reaction mixture (Wang et al., 1997; Loprete and Hill, 2002). After 3h incubation at 28°C, 2ml of reaction mixture was added with the 2 ml of dinitrosalicylic acid

(DNS) and boiled for 10 min. Absorbance were recorded at 575 nm, where DNS reagent alone was used as blank and glucose was used as standard. The enzyme activity was expressed as specific activity (SA) units. One SA is equal to µg glucose released per ml per hour.

Statistical analysis

Data was subjected to statistical analysis. Each parameter was analyzed separately by using one way of variance (ANOVA) with SPSS (t test) package. Student's t-tests were used to find out the level of significance at p≤ 0.05.

RESULTS

Fungal identification – morphological and molecular characterization

Colletotrichum gloeosporioides is a common plant pathogenic Coelomycetous fungus reported in causing fruit rot disease in *C. annuum* (Than et al., 2008). Based on the morphology of the mycelial colony and characteristics of the conidia, the fungus was identified as *C. gloeosporioides*. Fungal colonies grew well in PDA and good sporulations were obtained in 5–7 days of incubation (Figure 1A). Colony growth rate was recorded as 11–14 mm/day in PDA medium. The isolated fungus was identified with the salient features of culture morphology and conidial characters (Weir et al., 2012). The culture colony on PDA plate showed a pale brown or greyish white, consisting of hyaline, septate and branched mycelium. In culture, the fungus produced sclerotia, which are dark brown and occasionally setose. Setae are long, brown and septate. Conidiogenous cells are enteroblastic, phialidic, and hyaline. Conidia are hyaline, one-celled, straight, cylindrical and obtuse at apices measuring 9-23 × 3-4.6µm in size (Figure 1B). These are the salient features which confirm the identity of *C. gloeosporioides*, and highly distinguishable under phase contrast microscopy. Pathogenicity test of the fungus on the host plant further confirms the virulence property by showing the characteristic disease symptoms. The pathogenic nature of the fungus *C. gloeosporioides* based on pathogenicity test was also supported by an earlier report (Manandhar et al., 1995). Morphological features of the isolated fungus from the infected host were found to be similar compared with the inoculums (*C. gloeosporioides*). In addition to culture morphology, further confirmation was done by a molecular based method of fungal identification. From the molecular characterization, the 5.8s and 28S ribosomal RNA gene had a 594-bp fragment, which was amplified from the DNA of *C. gloeosporioides*. This sequence showed similarity with other fungus, *C. gloeosporioides*, GenBank No: JX010149) as revealed by nucleotide BLAST search. They were closely related by showing 99% of similarity between the strains.

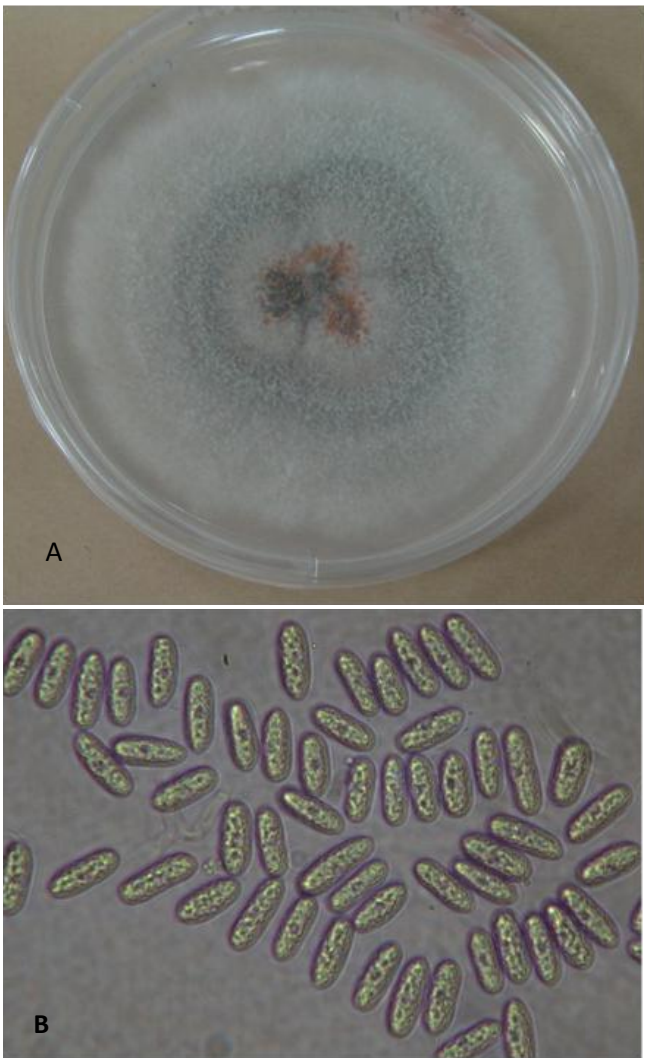

Figure 1.. A 7-day-old fungal culture showing the radial growth of the sporulated mycelia on PDA medium (A). Asexual conidia (400 X) of *C. gloeosporioides* (B) observed with phase contrast microscopy.

Enzyme activity

The inhibitory effect of plant extracts and dithane-M45 on the activity of PMG, PTE and CMC of *C. gloeosporioides* is expressed as the loss of viscosity in percentage and as relative and specific activity units (Figures 2, 3 and 4, Table 1). All the treatments (plant extracts and dithane M45) were significantly different at 1% level in comparison with control.

PMG activity

The highest percentage of viscosity loss (maximum of the enzyme) was found in the enzyme source with the plant

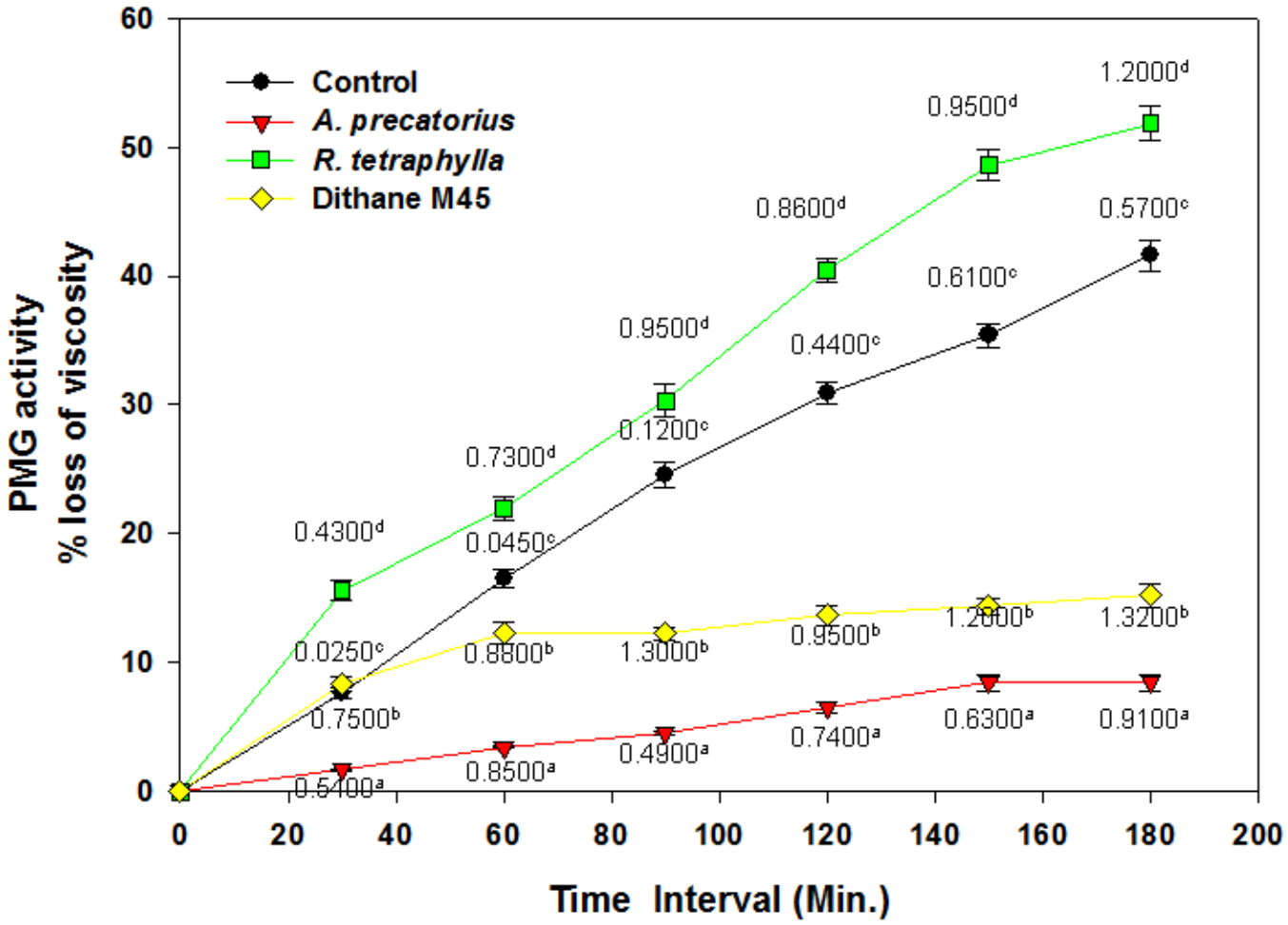

Figure 2. Effect of plant extracts and chemical fungicide (Dithane M45) on the polymethyl galacturonase (PMG) activity of *C. gloeosporioides* was estimated by the viscometric method using 1% pectin as a substrate in sodium acetate buffer (pH 5.2). Percent loss of viscosity was calculated for every 30 min. in a period of 3h. The plant extract and fungicide treatments were highly significant in comparison with control. Each value represents a mean of three replicate. Mean standard error bar (± SE) in the line followed by the different letter is significantly different according to the t-test (α= 0.05).

extracts of *R. tetraphylla* (51.85%) at 180 min. Least activity was recorded in the enzyme source treated with the extracts of *A. precatorius* (8.40%) which was far less when compared to that of dithane-M45 (15.23%) and control (41.58%) (Figure 2). The results in relative activity units showed that the highest rate of inhibition was present with the extract of *A. precatorius* followed by dithane - M45 when compared to the control and *R. tetraphylla* treatments (Table 1). The plant extract and fungicide treatments were highly significant at 1% level when compared with the control.

PTE activity

PTE activity was found to be the maximum in the enzyme source treated with the extracts of *R. tetraphylla* (31.34%) and minimum with the extracts of *A. precatorius* (11.32%) when compared with dithane-M45 (21.39%) and control (26.33%) (Figure 3). Similarly, enzyme activity expressed in RA units and SA units also showed that the activity of PTE was maximum in *R. tetraphylla* treated with *C. gloeosporioides* but were minimum in *A. precatorius* treatment followed by dithane-M45 and control (Table 1). The plant extract and fungicide treatments were highly significant at 1% level when compared with the control.

CMC activity

CMC activity at 180 min was found to be maximum in *C. gloeosporioides* treated with the extracts of *R. tetraphylla* (79.55%) and minimum with *A. precatorius* (7.35%) when compared with dithane-M45 (16.25%) and control (61.48%) (Figure 4). Similarly the RA units and SA units showed

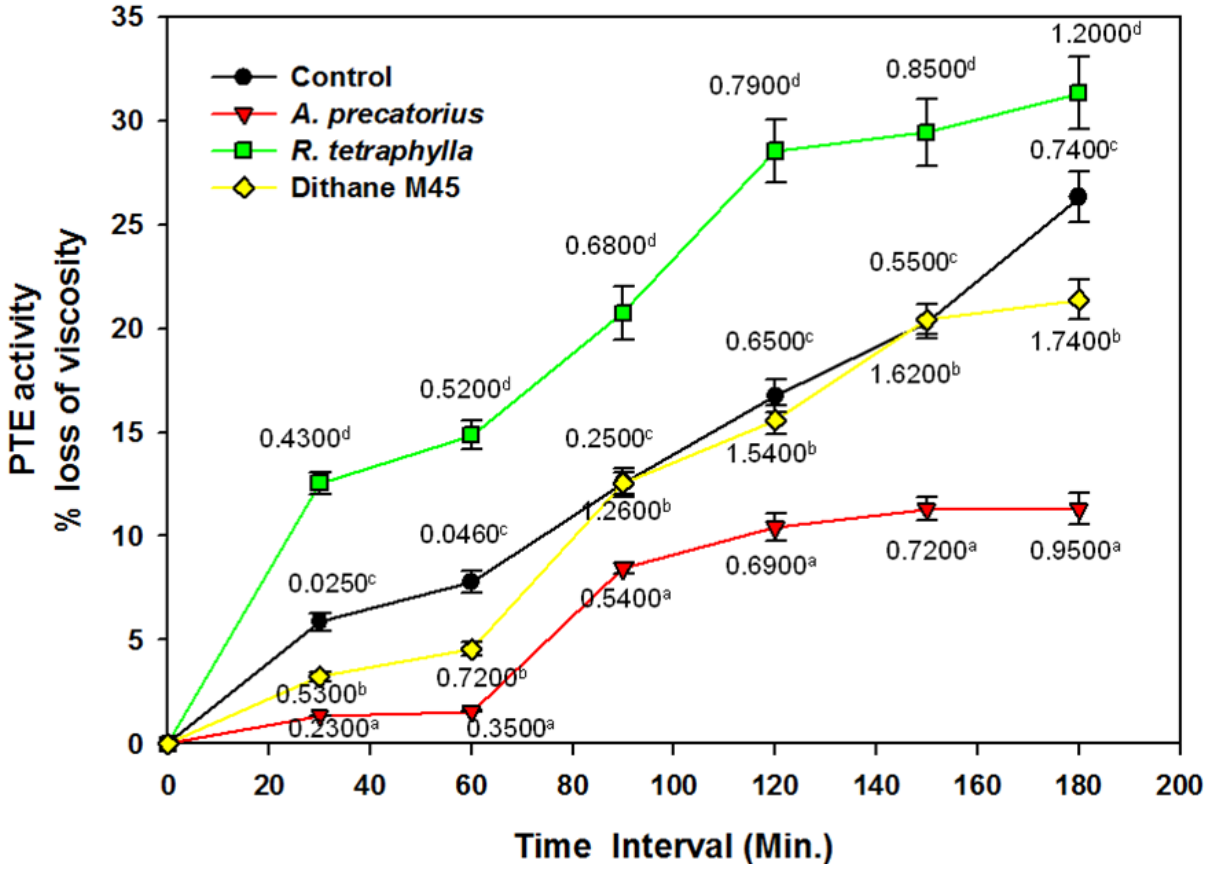

Figure 3. Effect of plant extracts and chemical fungicide (Dithane M45) on the pectin trans eliminase (PTE) activity of *C. gloeosporioides* was estimated by the viscometric method using 1% pectin as a substrate in boric acid-borax buffer (pH 8.7). Percent loss of viscosity of the reaxtion mixture was calculated for every 30 min. in a period of 3h. The plant extract and fungicide treatments were highly significant in comparison with control. Each value represents a mean of three replicate. Mean standard error bar (± SE) in the line followed by the different letter is significantly different according to the t-test (α= 0.05).

that the maximum activity was found in the *C. gloeosporioides* treated with the extracts of *R. tetraphylla* and minimum in *A. precatorius* followed by dithane-M45 and control (Table 1). The plant extract and fungicide treatments were highly significant at 1% level when compared with the control.

Of all the treatments, the activity of PMG and CMC was found to be maximum in the extracts of *R. tetraphylla* and minimum in *A. precatorius* treatment when compared with dithane-M45 and the control. All the treatments were highly significant at 1% level when compared with the control (Table 1).

DISCUSSION

Ethanolic root extracts of *A. precatorius* was found to inhibit both the endo and exo activity of PTE and CMC and only endo activity of PMG of *C. gloeosporioides* under *in vitro* condition. This might be due to the presence of phenolic compounds (polyphenols, phenolic acids, coumarins) in the extracts. Earlier work of Srivastava et al. (2013) supports the finding where the polyphenols, phenolic acids, coumarins of plants were found to inhibit the growth and enzyme production (ligninolytic and pectinolytic enzymes) of *Botryosphaeria* spp. Bride et al. (1960) has also reported that the varietal resistance of certain varieties of cider apples to brown rot was due to their high content of polyphenols. Inhibitory activity in PMG, PTE and CMC of *C. capsici* by the flower extracts of *Datura innoxia* under *in vitro* condition was reported earlier (Chitra et al., 2001). This also supports the presence of phenolic compounds (polyphenols, phenolic acids, coumarins) in the plant extract which cause the inhibition of enzymes activity.

Hedge and Podder (1997) have isolated cytotoxic lectin and abrin, a proteinaceous active principle from the roots of *A. precatorius* extract. The inhibitory effect of *A. precatorius* extract on the enzyme production and activity of *C. gloeosporioides* might also be due to the protein (ac-

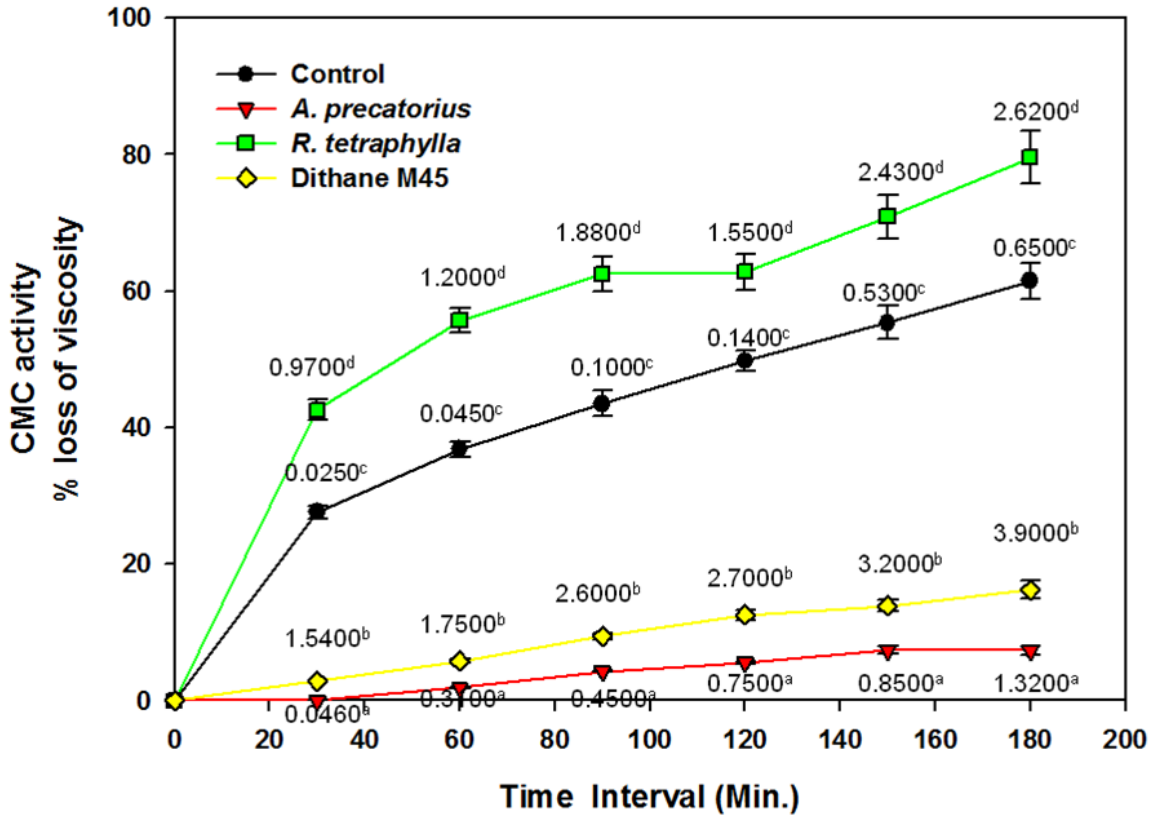

Figure 4. Effect of plant extracts and chemical fungicide (Dithane M45) on the carboxy methyl cellulase (CMC) activity of *C. gloeosporioides* was estimated by the viscometric method using 1% carboxy methyl cellulose as a substrate in sodium acetate buffer (pH 8.7). Percent loss of viscosity of the reaxtion mixture was calculated for every 30 min. in a period of 3h. The plant extract and fungicide treatments were highly significant in comparison with control. Each value represents a mean of three replicate. Mean standard error bar (± SE) in the line followed by the different letter is significantly different according to the t-test (α= 0.05).

(active principle) present in the root extracts. Gao and Shain (1995) reported that protein extracts from Chinese Chestnut inhibited the polygalacturonase production and activity in *Cryphonectrya parasitica* and also in *Colletotrichum lindimuthianum*. Apart from protein, Chacko et al. (1995) have isolated five isoflavanquinones from the root extracts of *A. precatorius* called abruquinones (A, B, C, D, and E). The above findings suggest that higher inhibition on the PMG, PTE and CMC of *C. gloeosporioides* that is 80.15, 57.9 and 88.5% respectively by *A. precatorius* root extract over control might be due to synergistic effects of active principles including flavonoids, phenols and proteins present in the root extracts.

Root extract of *A. precatorius* was more efficient in inhibiting the activities of all the tested cell wall degrading enzymes of *C. gloeosporioides* compared to dithane-M45 (chemical fungicide) and control. *R. tetraphylla* extract did not show any inhibition on the enzymatic activities of *C. gloeosporioides*. This might be attributed to the variation in the compounds present in the plant. Though the root extract of *R. tetraphylla* was found to inhibit both conidial germination and mycelia growth of *Colletotrichum* sp. (Kumaran et al., 2003), it was now found to be ineffective towards cellulolytic and pectinolytic enzyme activity. Similar results were also reported earlier. Aqueous leaf extracts of *Solanum torvum* and *Datura metal* inhibited both the conidial germination and mycelia growth of *C. capsici* but failed to inhibit the production and activity of polygalacturonase under *in vitro* condition (Gomathi et al., 2001). Komaraiah and Reddy (1986) also reported differential action of fungicides towards cell wall degrading enzymes. Dithane M-45 inhibited cellulolytic enzymes of *Corynespora cassicola* but copper oxychloride stimulated the activity of the same.

Out of the two extracts tested in the present study, *A. precatorius* inhibited the production and activity of cell wall degrading enzymes, on contradictory *R. tetraphylla* extract induced the enzyme activity. Inhibition of cell wall degrading enzymes is one of the modes of action as seen in the case of *A. precatorius*. The mode of action by inhibition of cell wall degrading enzymes was reported in only few fungicides (Hewitt, 1999, Yang et al., 2011). On the contrary *R. tetraphylla* did not inhibit the cell wall degrading enzymes and its mode of action might be

Table 1. Effect of plant extracts and Dithane M45 on the relative and specific activity of pectinolytic and cellulolytic enzymes of *C. glososporioides*. The plant extract and fungicide treatments were highly significant at 1% level when compared with control.

Treatment	PMG activity		PTE activity			CMC activity		
	RA	Viscosity loss at 180 min (%)	RA	SA Amount of TBA reacting substances produced	Viscosity loss at 180 min (%)	RA	SA* Amount of reducing sugar liberated	Viscosity loss at 180 min (%)
Control	10.976 ±0.4[a]	41.58 ±0.9[b]	5.890 ±0.085[a]	1.944 ±0.18[a]	26.33[b] ±0.57	37.233±0.84[a]	0.07630 ±0.0045	61.48 ±2.35[b]
A. precatorius	0	8.40 ±0.35[a]	0	1.720 ±0.065[a]	11.32 ±0.47[a]	0	0.04770 ±0.0063	7.35[a] ±0.25
R. tetraphylla	24.717±1.9[b]	51.85 ±2.43[b]	9.140[b] ±0.34	2.040 ±0.15[a]	31.34 ±1.23[b]	54.380 ±2.88[b]	0.57083 ±0.043	79.55 ±3.84[b]
Dithane -M45	0	15.23[a] ±0.58	0	1.940 ±0.174[a]	21.39 ±0.73[a]	0	0.05435 ±0.0045	16.25 ±0.62[a]

Means ± SE in column followed by the same letter are significantly different as determined by t test at 1% level; RA: Relative activity units represent the reciprocal of time in min. for 25% loss in viscosity X 10^3; SA: Specific activity units represent the changes in the absorbance at 575 nm (µg glucose/ml/hr); SA*: Specific activity units represent the changes in the absorbance at 575 nm of 0.001/ml enzyme/h.

different and should be investigated further.

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