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Ecofriendly Response of Citrus Peels to Alternaria Leaf Spots of Tomato: Exclusive Role of Peel Phenolics

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Abstract

Citrus is an important agricultural commodity of Pakistan. All of its uses including food and beverage industries result into the waste of its peel, which may be a remarkable reservoir of plant defensive biochemicals including phenolics; and can be used to control various plant pathogens e.g. *Alternaria alternata* causing leaf spots of tomato. Present study concentrated upon phenolics recovery and evaluation of antifungal potential of phenolics isolated from peels of various citrus types i.e. Lemon, Kinno, Malta, Mossambi and Feutrell's early. Maximum phenolics were recovered from Mossambi (0.21%) with the decreasing trend towards Malta (0.19%) > Feutrell's early (0.14%) > Lemon (0.13%) > Kinno (0.06%). Mossambi contributed better control of *A. alternata* in comparison with Malta, while the growth inhibition was less in case of Lemon, Kinno and Feutrell's early. Spray of phenolics prior to infection provided protection from fungal pathogen with a maximum disease in case of 1% Mossambi. A decreased curative potential of phenolic acids prevailed in order: Mossambi > Malta > Feutrell's early > Kinno > and Lemon with the maximum individual percentage control of 73.4 > 59.7 > 54.7 > 45.5 > and15.3%; respectively. Such high antifungal potential of phenolics makes citrus peel an important source of pesticide.

Keywords: *Alternaria alternate;* Citrus peels; Curative and protective potential; Phenolics

Introduction

Plants always have been an important source of antimicrobial chemicals since the humans have been trying to control pathogenic diseases using antibiotics. A number of antibiotics have been isolated up till now. But the demand for new antibiotic formulations has also been ever increasing due to acquired resistance of microbes against designed antibiotics. Microbes have the ability to acquire and transmit resistance in other organisms (Gislene *et al*., 2000). Thus, there is a continually increased pressure to find novel antimicrobial chemicals. New drugs and medicines can be developed by studying these compounds naturally produced around us (Verma and Dubey, 1999; Cragg *et al*., 1999). Plants are rich in several phytochemicals, which are known to play important roles in plant metabolism and defense against several diseases.

Among different compounds of plant origin, phenolics are of the most widespread and are of great significance in plant development. These compounds protect host against bacteria, fungi, herbivores, insects and viruses that plague the plant (Duke and Bogenschutz, 1999). It is therefore important to discover natural antibiotic formulations for obtaining a sustainable control of microbes. These compounds have potential for control of fungal diseases of crops (Cutler and Hill, 1994). The prospect of using these compounds for the development of natural fungicides is appealing, because most of these compounds are readily available, environmentally safe, less risky for developing resistance in pests, less hazardous to non-target organisms and pest resurgence, has less adverse effect on plant growth, less harmful to seed viability and quality, and above all, less expensive (Prakash and Rao, 1997; Javaid *et al*., 2010; Zaheer *et al*., 2012).

Phenolics have been used against a number of microbial diseases and may be potentially used against devastating fungal pathogen *A. alternata* in case of tomato. Species of *Alternaria* have been significantly controlled with the phenolics of olive oil pomace (Eleonora *et al*., 2005) conferring the antifungal activity of phenols against genus *Alternaria*. On the basis of this evidence phenols can be confidently exploited for the management of *A. alternata* in tomatoes.

There are a number of sources in our surroundings for obtaining phenolics but in the present study citrus has been selected. Citrus family is included among the plant families that contains relatively higher amount of phenolic compounds in different parts of plants (Zaat *et al*., 1987; Laks and Pruner, 1989; Snyder and Nicholson, 1990). Polymethoxylated flavonoid is a well-known group of secondary metabolites obtained from citrus species. The phenolics that occur in citrus include flavonoids (flavanones, flavones, and flavonols), anthocyanins, coumarins, and psorolens, amongst others. In addition,

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phenolics of citrus appear to have desirable antimicrobial properties. Some have been reported to be antifungal agents and to exhibit antiviral and antibacterial activities (Robbins, 1980).

As a result of high antifungal activity of citrus phenolics, the aim of present study was to evaluate the *in vitro* antifungal activity of citrus fruit varieties viz. Lemon, Malta, Kino, Mossambi and Feutrell's early against *Alternaria* spp., the main cause of leaf spot diseases of tomato.

Materials and Methods

Procurement of Pathogen

Pathogenic culture of *Alternaria alternata* was procured from First Fungal Culture Bank of Pakistan (FCBP) and then maintained on 2% malt extract medium for getting it refreshed and subcultured after one month.

Pathogenicity Test

The pathogenicity test was performed according to Grogan *et al*. (1975). Two kg of soil was sterilized at 45°C for 24 h in hot air oven. Culture was grown at 26 ±2°C for about 10 days. Conidial suspension of *A. alternata* was prepared according to French and Hebert (1982). Conidia from the surface of 10 days old culture plates were harvested by suspending them in distilled water. The stock suspension was serially diluted to prepare conidial suspension of 2.0×10^5 conidia/mL with haemocytometer and used as inoculum.

Inoculation of Soil and Aerial Plant Parts

Pathogenicity test was performed by spraying one month old tomato plants to runoff with spore suspension containing approximately 2.0×10^5 conidia/mL. The plants were kept covered with polythene bags for 48 h to maintain sufficient moisture for spore germination and development of disease and after that the plants were returned to the greenhouse. Greenhouse temperature ranged from 30°C to 35°C.

Disease Rating Scale

Disease rating scale was made on the basis of disease incidence and disease severity. Disease incidence was observed as the symptoms appeared on the plant and disease index was calculated with the help of following formula:

$$Disease\ index = \frac{Commulative\ number\ of\ observed\ parts\ acc.to\ DRS \times 100}{Maximum\ scale\ number \times Total\ number\ of\ observed\ parts}$$

Isolation of Phenolics

The phenolics were isolated according to the protocol of Mujica *et al.* (2009). Citrus peel was collected from the fruit juice shops and other factories of district Lahore and was brought to Fungal Biotechnology Lab. All types of peels were oven dried at 45°C for three days and powdered. Then pre-weighed 50 g powder was dipped into 80% acidified methanol solution overnight and then vacuum dried at 45°C to get phenolics. Data were recorded in terms of percentage of phenolics recovered from each peel type.

$$Percentage\ phenolics\ recovered = \frac{\text{Mass of phenolics obtained}}{\text{Mass of plant material taken}} \times 100$$

Application of Phenolics

Phenolics of different citrus types were dissolved into DMSO (100 mL) to get stock solutions of 50%. Each stock solution was further diluted into 1, 2, 3, 4, 5, 6 and 7% solutions, which were analyzed for their antifungal potential against *A. alternata*.

Antifungal Assay

All concentrations of phenolic solutions were added up with 2% malt extract and agar. Among the treated media a control was also established with 2% Malt Extract Agar medium prepared in distilled water. Media were poured into 9cm Petri plates and 7 days of incubation (at controlled conditions) were provided to each plate after inoculating with *A. alternata*. Each treatment was established in triplicates. After incubation period diameter of each colony was measured. Data were recorded in terms of percent growth inhibition calculated by the formula:

$$\%age\ growth\ inhibition = \frac{\text{Diameter of control} - \text{Diameter of treated plate}}{\text{Diameter of control}} \times 100$$

Detached Leaf Assay

Tomato leaves were detached from parent plant and placed into sterilized Petri plates (already floored with two filter papers in each plate) in such a way that petiole ends were inserted into filter papers. Filter papers were moistened with 2 mL of distilled sterilized water and leaves were inoculated with 0.5 mL of pathogen inoculum (1500 spores/mL). Petri plates were incubated at 25±2C for seven days and data were recorded as disease progress rate. Protective potential of citrus phenolics: Tomato plants were grown in pots under the controlled environment of green house and at the age of one month they were sprayed with all five types of citrus phenolics of different concentrations i.e., 1, 3, 5, 7 and 9% (Tice *et al*., 2000). After 6 h fungal inoculum of 1500 spores/mL was also applied to same plants and water was supplied on the basis of need. After the incubation period of two weeks disease symptoms, percentage of disease index and percentage control was recorded.

$$Percentage\ control = 100 - Percentage\ disease\ index$$

Curative Potential of Citrus Phenolics

Tomato plants of one month of age were sprayed with fungal inoculum (1500 spores/mL) under the green house conditions. Pathogen was allowed to establish for four days prior to phenolics spray of various concentrations. After 10 days of incubation disease index and percentage control was calculated for each concentration.

Statistical Analysis

Standard errors of means of three replicates of each treatment were calculated. All the data was analyzed by analysis of variance (ANOVA) using computer software SPSS. Following the ANOVA, Duncan's Multiple Range (DMR) test (Steel and Torrie, 1980) was applied to separate the treatment means using Computer software COSTAT.

Results

Hypersensitivity and Pathogenicity Assay

A. alternata readily infected the tomato plants under controlled environment when applied to four tomato varieties widely cultivated in Lahore, Pakistan. Infection was evident in 7-8 days, and within 10-15 days, the stem and leaf spots enlarged, eventually cankers were formed resulting in death of susceptible plants (Fig. 1). Dinaar was proved the most susceptible variety for *A. alternata* with 13.9% DI. The descending order of DI among other tomato varieties prevailed from Jeury > Mailsi > Sahil with DI of 10.03 > 8.15 > and 5.06%, respectively (Table 1). Thus, Dinaar was selected for downstream experiments due to its high susceptibility for fungal pathogen.

Phenolics Isolation

Maximum amount of phenolics (0.21%) recovered from peel concluded Mossambi to be prominent among all other types of citrus (Table 2), while the second most efficient was Malta peel from which 0.19% of phenolics were isolated. The quantities of phenolic acid components among the rest of citrus peel were fruiter (0.14), lemon (0.13) and kinno (0.06).

Fungal Growth Inhibition

Antifungal bioassays conducted with peel extract revealed that there were two types of phenolics (Mossambi and Malta) whose 7% concentrations completely inhibited fungal growth. Mossambi provided complete control of fungus even at lower (6%) concentration (Fig. 2). Two more concentrations viz. Mossambi 5% and Malta 6% possessed growth inhibition of 92.7% and 94.6%, respectively. Other two concentrations followed the above concentrations with significant differences were Malta 5% (83.2%) and Feutrell's early 7% (70.3%). In spite of belonging to same group of plants (citrus) On the other hand, Malta, Mossambi and Feutrell's early also inhibited the growth. The overall trend of fungus growth inhibition among citrus peel phenolics was in order of Mossambi > Malta > Feutrell's early > Kinno > and Lemon (Fig. 2).

Table 1: Routinely cultivated tomato varieties with their respective disease index

Tomato variety	Disease index
Sahil	5.06
Dinaar	13.9
Jeury	10.03
Mailsi	8.15

Table 2: Percentage recovery of phenolic acids from different citrus peels

Citrus	Percentage phenolics recovered
Lemon	0.13±0.03
Kinno	0.06±0.01
Malta	0.19±0.07
Musammi	0.21±0.05
Fruiter	0.14±0.01

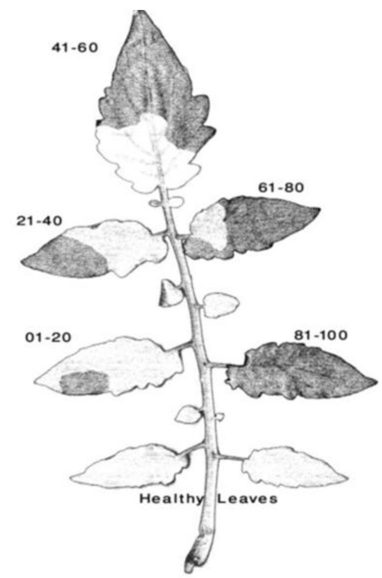

Fig. 1: Visual explanation of disease rating scale

Disease Progress Rate

In the detached leaf assays, disease symptoms started to develop just after inoculation. After 24 h of incubation some leaves of Petri plates exhibited minute spots and started showing the development of symptoms with 3.7% of infected area. At the 2nd day symptoms were fully established and leaves covered about 7.8% area of leaves. With the progressing time from 2nd to 5th day, slope of disease progress curve was very sharp and 90.3% leaf area was infected on 5th day. Till sixth day 98.4% leaf area was infected (Fig. 3).

Protective Characteristics of Phenolics

An experiment was designed to determine the protective nature of isolated phenolics against the target pathogen as shown in Fig. 4. Results showed that phenolics completely protected the tomato plants from fungal pathogen; using

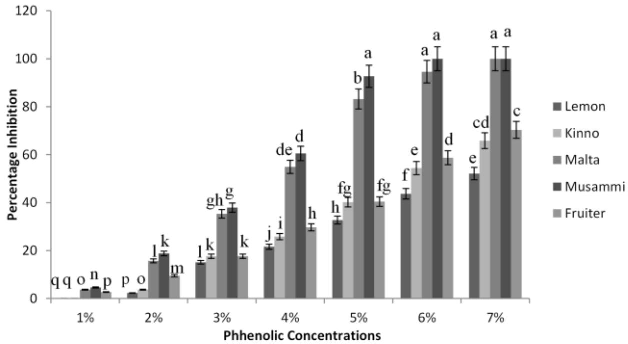

Fig. 2: Percentage growth inhibition by phenolic contents

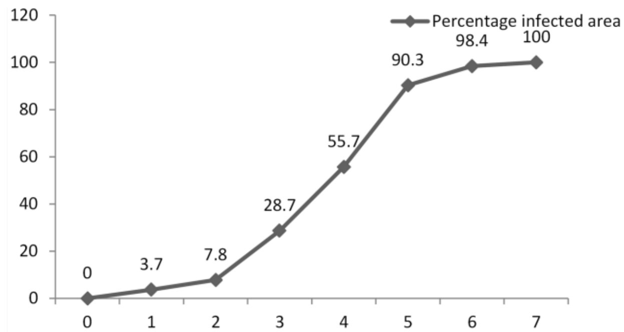

Fig. 3: Determination of disease progress curve by detached leaf assay

Mossambi (9%), followed by Malta (9%) peel extract with significant difference. Lower concentrations of Lemon, Kinno and Malta were not proved effective to protect tomato plants from *A. alternata* infection (Fig. 4), in contrast to this, the other two citrus types i.e., Mossambi and Feutrell's early induced 9±0.3 and 2.8±0.6% disease control; respectively (Fig. 4D and E). The complete analysis of protective activity revealed that application of Mossambi phenolics was the best than those of phenolics was the least effective among all strategies studied.

Curative Activities of Phenolics

Maximum curative activity was again performed by Mossambi at 9% concentration; it was continuously increased from 10.2 to 73.4% when phenolics concentrations were increased from 1% to 9%, respectively (Fig. 5). Curative ability of Malta was evaluated as second among all the citrus types used as its highest concentration (9%) gave 59.7% control of fungal pathogen; while, its least concentration (1%) was unable to control pathogen growth (Fig. 5C). Following this, 9% phenolic concentration of Feutrell's early and Kinno exhibited 54.7 and 45.5% curative potential against the disease, respectively (Fig. 5B and E). Results revealed an interesting fact about Feutrell's early and Kinno as the higher phenolics concentration of Feutrell's early gave better arrest of growth of *A. alternata* than the respective phenolics concentrations of Kinno. But, at lower concentrations Kinno delineated as better controlling agent because even its least concentration controlled fungal growth up to 2.1%; while the least concentration of Feutrell's early phenolics produced no fungal control. The least efficient control was of Lemon which produced only 15.3% control at the highest concentration used (9%).

Discussion

Phenolics are procured from natural resources and are used to perform a lot of functions i.e., preservation of colors, flavors, protection from vitamins degradation, production of pigments, growth reproduction antimicrobial activities etc. (Lattanzio *et al*., 2006). There are a number of different modified phenols extraction methods with varying phenol recovery values; and still researchers are keen about finding out the best sources and their concentrations (Tsakona *et al*., 2012). There are a number of studies showing that phenolics are mainly dependent upon pre-extraction method of peel preparation, solvent used for extraction, temperature conditions and type of citrus peel used (Manthey and Grohmann, 2001; Li *et al*., 2006; Hayat *et al*., 2010). Present study is the reminiscent of all the previous studies because citrus peel from different sources not only produced varying amounts of phenolics but antioxidant abilities of phenolics obtained were also not the same. Diankov *et al*. (2011) concluded that extraction method not only affected the amounts of phenolics but it also influenced the antioxidant properties of phenolics procured.

Phenolics exhibit significant antifungal properties and have been found to successfully retard fungal growth in many investigations (Marsilio *et al*., 2001; Esekhiagbe *et al*., 2009). All the above mentioned studies, different methods for measuring the growth reduction of fungi were used and all methods concluded the same antifungal behavior of the phenols. All of antimicrobial activities of polyphenols are due to their antioxidant properties (Cetin-Karaca, 2011; Salawu *et al*., 2011). Presently, a significant reduction in growth of *A. alternata* argues upon antifungal activity of polyphenols as well as strongly supports previous studies.

Copping and Hewitt (1998) conclude that phenolics have no target specificity and act upon highly unselective objects might be resulting into a threat to many environmental factors and ultimately humans. So, releasing high amounts of intact phenolics reservoirs in environment may be harmful. Hence, they should be isolated and used for some beneficial purpose in order to reduce environmental threats. Present results also showed that antifungal activity of phenolics against a vast range of fungal species without any specific target. So, it is highly recommended to extract these novel biochemicals from fruit peel before going it wasted. It will not only keep our environment protected but also provide our crops rid from destructive pathogens.

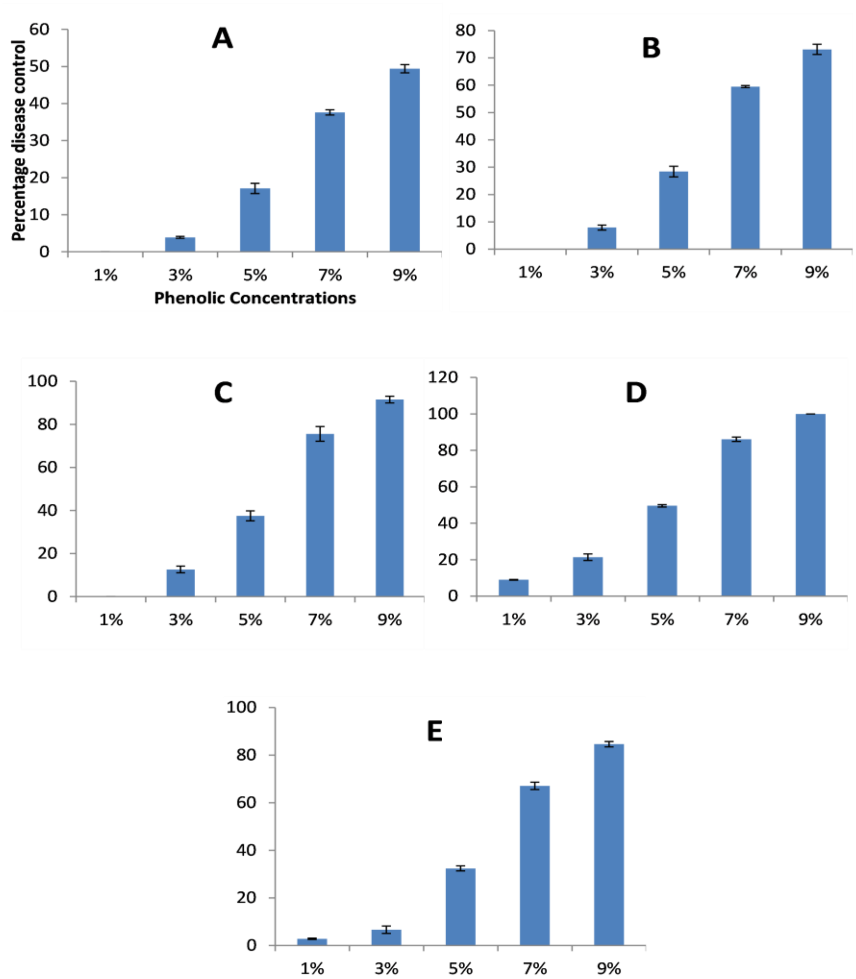

Fig. 4: Protective activity of citrus phenolics against *A. alternata* tomato leaf spot. Efficacy of phenolics obtained from Lemon (A), Kinno (B), Malta (C), Musammi (D), Fruiter (E)

Phenolics are also involved in the protection of plants from ultraviolet rays and oxidants, which cause great damages to plants and make plant liable for fungal infections (Lattanzio *et al*., 2006). This shows that antioxidant property of phenols works for the benefit of plant. So, plants have to use phenolics for their survival against such diversified enemy community. Moreover, the phenolics also provide protection duties significantly, if applied to plants externally or generated by plants themselves.

Plants have to produce more defensive compounds than animals because due to lack of locomotion; which sneeze the option of locomotory escape from plants. So, they have to survive against infectious entities just on behalf of defensive elements (Bell, 1980). Meanwhile, "Higher the concentration of phenolics greater will be the anti-pathogenic protection" is a common hypothesis of plant protection studies (Esekhiagbe *et al.*, 2009). It is also notable that plant have maximum phenolics contents at the most important stages of their development e.g. reproduction (Paiva, 2000; Pichersky and Gang, 2000; Lattanzio, 2003a, b). So, this study supports the hypothesis that increased tissue phenolics gave better and efficient control of pathogenic infection.

In conclusion, the phenolics are the precious blessings of nature to plants. Artificially enhanced quantities of these defense weapons may enable plant to stand against more devastating pathogens. So, artificial formulations of phenolics will be helpful in plant disease control.

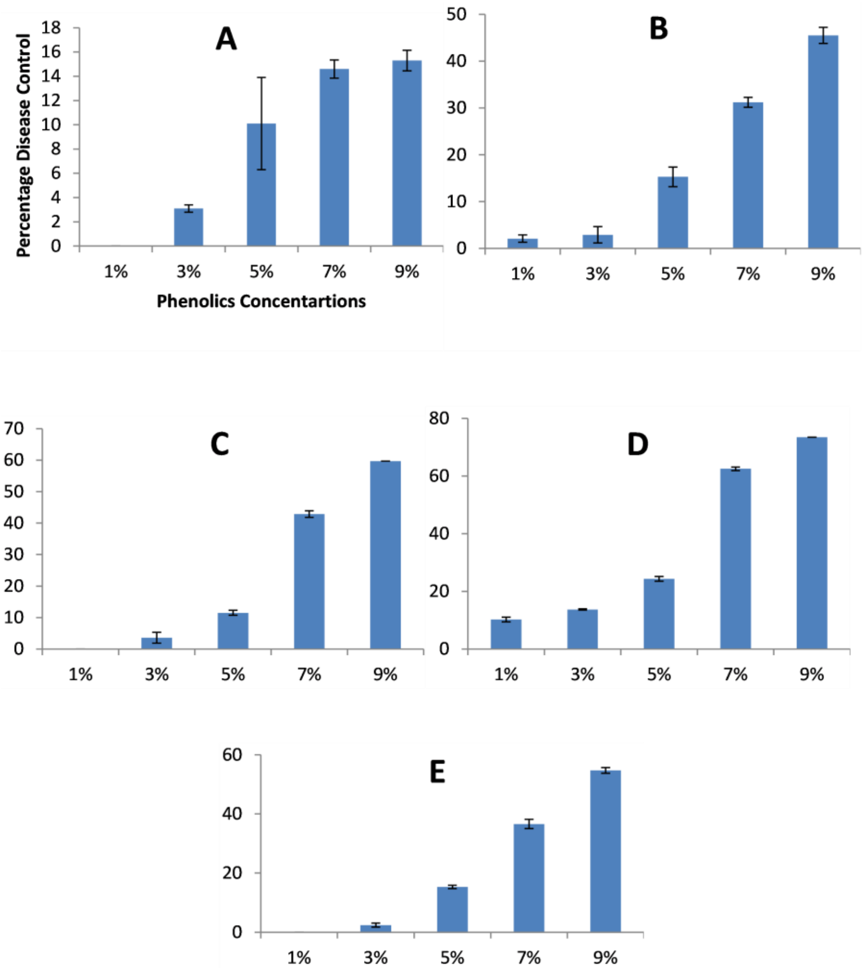

Fig. 5: Curative activity of citrus phenolic acids against *A. alternata* tomato leaf spot. Efficacy of phenolics obtained from Lemon (A), Kinno (B), Malta (C), Musammi (D), Fruiter (E)

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