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Molecular detection of *Phytophthora Capsici* from Chilli (*Capsicum annum L.*) and its response to selected growth media and vegetable extracts

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Abstract

The present study was conducted to evaluate selected culture media for rapid multiplication of *P. capsici* and its *in-vitro* control through the use of phytobiocides. Another aspect of this research was to detect the fungus in diseased plant material by molecular method. Significant differences ($P \leq 0.05$) were observed among the three culture media i.e. Potato Dextrose Agar (PDA), Corn Meal Agar (CMA) and Water Agar (WA) for radial colony growth, sporangial production/germination and biomass production of *P. capsici*. PDA proved to be the best medium in having maximum colony growth, the highest biomass production and 95% spore germination in the least possible time. Among the five different phytobiocides having tested, extracts from garlic, cabbage and peppermint retarded the mycelial growth while those from onion and radish stimulated the pathogen development. *P. capsici* was successfully detected through PCR in symptomatic and asymptomatic albeit diseased plant tissues when 595bp fragment of fungal genome was amplified by *P. capsici* specific primers.

Keywords: *Phytophthora capsici*, Culture media, Molecular detection, Phytobiocides

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1. Introduction

Chilli (*Capsicum annum* L.), one of the most important vegetable, is used both as salad and dried condiments. It is important as fresh and cooked vegetable throughout the world (*e.g.* pickles and spices etc) (Bosland and Votava, 2000). It is also grown for ornamental purposes, especially for its fruit with bright and attractive colors (Cronin, 2002). It is rich in vitamins A, C and B complex, iron and calcium as well as magnesium, phosphorus and sulphur. It has an enzyme, capsaicin, which helps in digestion of food, prevents heart diseases by dilating blood vessels (Collins and Bosland, 1994).

In Pakistan, an area of 47.3 thousand hectares is under chilli cultivation producing 69.7 thousand tons of chilli. In Khyber Pakhtunkhwa (KP), area under chilli cultivation is 0.6 thousand hectares producing 0.7 thousand tons of chilli (MINFAL, 2006-07). According to official statistics, Pakistan exported red chilies to Middle East, USA and other European countries and earned Rs. 192.7 million during 2004-05 (MINFAL, 2005-06).

Chillies are prone to be attacked by a multitude of diseases which cause approximately 33% yield loss. Root rot, a destructive soil borne disease, caused by an oomycete *Phytophthora capsici* (Leonian), has become a serious threat to successful chilli production and causes upto 100% yield losses in commercial chilli fields (Babadoost and Islam, 2003; Babadoost, 2004). It becomes a severe problem during periods of heavy rainfall; the pathogen spreads quickly within the crop and results in heavy losses within a short period of time (Kim et al., 1997). In Pakistan, the disease is prevalent in chilli producing areas including KP (Hassan, 1994; Mehaboob et al., 1998) and Punjab (Saleem et al., 1997).

Despite its importance and associated losses, research on this devastating disease has been sketchy. Many areas including biology, epidemiology, molecular techniques and management of this disease need to be addressed. The pathogen is soil borne and is mostly cultured on a selective medium (PARPH medium) to avoid contamination (Papavizas et al., 1981). However, the selective medium can not be used in routine laboratory work as it is very expensive, its ingredients particularly the antibiotics are not easily available in the market and furthermore, it allows slow growth of the fungus. It is therefore, important that inexpensive nutrient media shall be investigated to grow the fungus under normal laboratory conditions.

Additionally, development of a quick and exact method of detecting the pathogen would be helpful in preventing the disease as well as taking timely management decisions and avoiding environmental contamination due to excessive use and abuse of chemicals. Up till now, detection methods comprised of visual examination, on the basis of taxonomic key (Stamps et al., 1990) and isolation in selective media, but these conventional techniques are time- consuming, laborious and require expertise of microbial (fungal and bacterial) taxonomy. Molecular techniques, particularly the polymerase chain reaction (PCR), have successfully been employed in detecting and identifying fungal plant pathogens (Lacourt and Duncan, 1997; Schena et al., 2002 a, b).

As regards the management of the disease, it is really difficult to control, due to its wide host range and its ability to survive in soil. Even a single chilli variety with resistance to *P. capsici* is not available to help control this menace. The existing management strategies include avoiding fields with a history of disease, growing

crops in well-drained soils, following regular crop rotation practices to avoid other susceptible crops (tomatoes, eggplant, and other cucurbits), and the application of different fungicides. However, all these strategies have failed in producing effective, economical control of the disease (Bosland and Lindsey, 1991). The use of phytobiocides which are rich in biologically active ingredients may prove better than the fungicides in effectively controlling this disease. Phytobiocides are environment friendly, easy to apply and relatively inexpensive. Moreover, the intercropping of plants with chilies in the same field shall be an additional source of income for the growers. Compared with the fungicides, these may also improve the cost benefit ratio and thus help in their popularity among the growers.

2. Material and methods

2.1. Molecular detection of *P. capsici*

2.1.1. DNA extraction from mycelium, pre-symptomatic and symptomatic Chilli plants

For extracting total genomic DNA from mycelium, the pathogen isolate was cultured in still pea broth (Cooke et al., 2000). Following a week of incubation at 24˚C, the mycelium was harvested and freeze-dried for extended storage at –20˚C. Similarly, plant material collected from a nearby farmers chilli field, was stored at 4˚C in plastic bags before further processing. For extracting DNA from infected plants and mycelium, samples were macerated under liquid nitrogen using a mortar and pestle. The powder was transferred to an Eppendarf tube to which distilled water was added to make the final volume to 3ml. A weighed amount of each material (0.5ml) including fungal mycelia and infected as well as healthy plant material was transferred to Eppendarf tubes, centrifuged at 7000 rpm at 4˚C for 10 minutes; supernatants discarded and pellets were re-suspended in 100µl of DNA Zole direct (Molecular Research Centre Inc. Cincinnati, Ohio, USA) solution. The mixture was left undisturbed at room temperature for 15minutes and was then saved at 4˚C for further use. An aliquot of 3µl (per 50 µl PCR volume) of the master mix was directly added as template DNA to the PCR tube.

2.1.2. Primers and PCR amplification

A specie specific primer pair for *P. capsici* (CAP primers) used:included,(CAPFW;5′TTTAGTTGGGGGTCT TGTACC3′), and (CAPRV2;5′TACGGTTC ACCAGCCCATCA3′) (Silvar et al., 2005) provided by D. Cooke, SCRI, Scotland, UK. Primers were from a region of high dissimilarity by comparison of different *Phytophthora* species (Cooke et al., 2000). PCR reagents for a single reaction (final volume of 50µl) were as follows.

Primer F (1µl), Primer R (1µl), dNTPs (5µl), DNA Taq polymerase (1µl), Taq buffer (4.5 µl), $Mgcl_2$ (4µl), Template (3 µl), Nuclease free H_2O (30.5 µl). PCR amplification was performed in a MJ mini thermocycler (Biorad, USA) with the following conditions: 95˚C for 5minuts (once. initial denaturation), 94˚C for 30sec. (denaturation), 55˚C for 30 sec. (primer annealing) and 72˚C for 50sec. (extension) repeated for 35cycles, and a final extension of 72˚C for 8min. (once). Following PCR amplification, 25µl from each reaction was electrophoresed through a 1 % (w/v) agarose gel (Sambrook et al., 1989).

2.1.3. Gel electrophoresis

PCR products were electrophoresed through 1% agarose (0.333g agarose dissolved in 30ml TBE buffer (Appendix II). Agarose was poured into gel electrophoresis tray and allowed to set at room temperature and TBE buffer was added in order to cover the top of the gel. The PCR product (25µl; after mixing with the blue tracking dye) of each sample was then loaded into the wells of the gel along with 1 kb DNA ladder for comparison. Electrophoresis was performed according to standard procedures (200 volts, 30minuts). The gel was then stained with ethidium bromide (0.5µg/ml) solution for 15minutes and destained in water for visualizing the amplified DNA. The stained bands were observed under UV light in UV tech machine and images were saved.

2.1.4. Fungal isolate and comparison of culture media

The isolate of *P. capsici* used for this study was obtained from Department of Plant Pathology; KP Agricultural University Peshawar which was Collected during a previous survey, also used for molecular detection. The pure culture of the isolate was also maintained on selective medium called PARPH (Pimiracin, Ampicilin, Rifampicin, PCNB, Hymexazol) for further use (Papavizas et al., 1981).

Selected culture media i.e., Potato Dextrose Agar, Water Agar and Corn Meal Agar were tested to compare their efficacy for radial colony growth, production of sporangia, germination of spores and biomass production of *P. capsici*. The experiment was laid out in completely randomized design (CRD) with five replications.

2.1.5. Radial colony growth

A number of different culture media i.e., Potato Dextrose Agar, Corn Meal Agar and Water Agar was tested for rapid multiplication of *P. capsici.* A 0.5cm plug of the test isolate was excised from the periphery of a colony of *P. capsici* and placed in the centre of each Petri plate under aseptic conditions. Plates were sealed with parafilm, appropriately labeled, and incubated at 25°C for two weeks. Radial growth of the fungus was measured by recording the colony diameter along two perpendicular lines and then taking the mean of the two measurements.

2.1.6. Production of sporangia

P. capsici isolate, grown on various culture media at 25 °C for one week was examined for the production of sporangia after one week the sporangia thus produced were harvested by flooding each Petri plate with 10ml sterile distilled water (SDW) and scrapping the plate surface with a sterile glass rod. The number of sporangia was counted by using a haemocytometre to determine their concentration per ml in order to make comparisons amongst various media.

2.1.7. Spore germination study

For spore germination experiment, the colony surface of a two week old culture of the pathogen grown on each of the test media was flooded with 5ml SDW and scrapped with a rubber spatula. The spore suspension

was filtered 4through cheese cloth to remove mycelial fragments and adjusted to a final concentration of 10 sporangia/ml. Aliquots (20µl) of the resulting suspension were then spread on each of the test media in Petri plates with a glass rod under aseptic conditions. Plates were incubated at 25°C. Number of germinated propagules in 15-20 microscopic fields (10x) was counted after every two hour interval until 95% sporangia germinated. A propagule was regarded as germinated if the germ tube was approximately twice the length of the sporangium. A minimum of 200 propagules/plate were counted.

2.1.8. Evaluation of phytobiocides in vitro

Freshly collected leaves of garlic (*Allium sativum L.*), peppermint (*Mentha piperita*), cabbage (*Brassica*º *oleraceae var. capitata*), radish (*Raphanus sativus L.*) and onion (*Allium cepa L.*) were dried for 2-4 days at 60˚ C to reach constant weight, and then finely ground. A 10 g sample of each plant material was placed in 100 ml of 99.5% ethanol (Merck) and left in dark overnight at room temperature, the extract was then heated at 40˚C for 4 hr in a water bath. The mixtures were filtered and sterilized. The ethanol solution was evaporated at 40˚C and the residue was dissolved in 2.5 ml of 99.5% ethanol. The extract was stored for further use at 4˚C (Fikret and Sara, 2006). The test isolate of *P.capsici* was plated on phytobiocides amended medium in Petri plates. Potato Dextrose Agar was used in this experiment as a medium because of its rich mycelial colony growth. Following autoclaving, each biocide was added separately into the medium after cooling @ 5ml/ 500ml. A 0.5cm plug of the test isolate was then placed in the centre of each Petri plate containing the amended medium under aseptic conditions. The plates containing the test isolate without any amendment were treated as control. The plates were sealed and incubated at 25˚C for two weeks, to allow fungal growth. Radial growth of the isolate was then assessed as described previously. Completely randomized design with five replications was followed in the experiment.

3. Data analysis

Data were analyzed through statistical software, MSTATC (Bricker, 1991) using one way Analysis of variance (ANOVA One) and Fisher's Least Significant Difference (LSD) test separated means when ANOVA one showed significant differences

4. Results

4.1. Molecular Detection of *P. capsici* in pre-symptomatic and symptomatic Chilli plants and mycelia of *P. capsici*

The primer set CAPFW/CAPRV2 gave the most consistent amplification of pure P. capsici DNA resulting in the production of 595bp product following optimization of PCR conditions including annealing temperature and time, primer extension time, concentration of primers, number of PCR cycles and gel concentration.

The primer pair CAPFW/CAPRV2 was used to identify fungal DNA in the plants. Using conventional PCR with this primer pair, effort was made to detect fungal DNA from infected symptomatic plant parts and

mycelia (DNA extracted from freeze dried mycelia of *P. capsici* which was used as template in PCR reactions) of *P. capsici.* When tested, strong amplification products of 595bp were obtained from mycelia of *P. capsici* and infected symptomatic plants (Figure 1).

Having tested the reproducibility of PCR assay to detect *P. capsici* in symptomatic tissues, the assay was used to detect infection in non symptomatic tissues. Amplification products produced from DNA extracted from symptomatic and non symptomatic plant tissues were of the same size. PCR products of 595bp were consistently obtained from such tissues. All reactions were repeated at least twice and the results were found to be consistent. Moreover, the assay was found sensitive to detect low levels of infections.

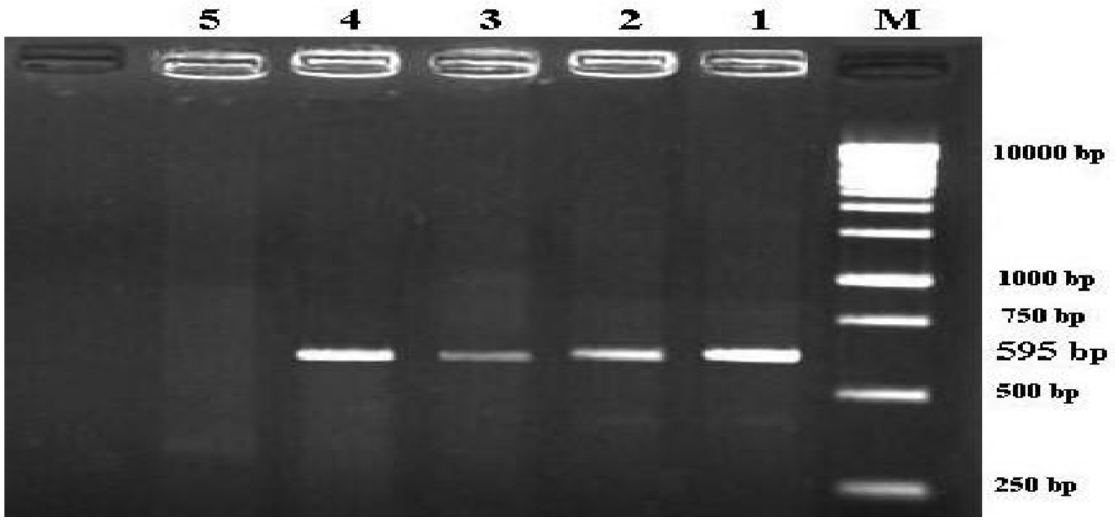

Figure 1. Amplification of *P. capsici* 595bp band from pre-symptomatic and symptomatic chilli plants and mycelia of *P.capsici*. M= Marker; Lane 1= Mycelia, Lane 2= Infected Plant Stem, Lane 3= Asymptomatic Plant Stem, Lane 4= Positive Control, Lane 5= Negative Control

4.2. Comparison of media for growth of *P. capsici*

Fungal growth parameters including radial colony growth, production of sporangia and sporangial germination were studied on three selected culture media.

4.3. Radial colony growth

Culture media used were significantly different ($P \leq 0.05$) from one another with respect to mycelial growth of *P. capsici* (Table 1). Among the three different culture media, Potato Dextrose Agar (PDA) medium was the best in supporting the radial growth of *P. capsici* with the highest colony diameter (3.49 cm) following two weeks of incubation. This was 24.92% more than the minimum growth observed on CMA. P DA was followed by

Water Agar (WA), which showed mean colony diameter of 3.21cm and was 18.38% more than that of Corn Meal Agar. The minimum mycelial growth of 2.62 cm was observed on Corn Meal Agar (CMA) medium.

4.4. Production of sporangia

There were -s1ignificant differences (P≤0.05) among the media used with respect to number of sporangia ml produced after one week of incubation. The pathogen produced maximum number of sporangia (26000/ml) on CMA (Table 1). This was 97.26% more than the minimum number of sporangia produced on W1A. PDA (1430/ml) ranked second to CMA in producing the maximum number of sporangia ml- which was 50.34% more than the minimum. The least number of sporangia was produced on water agar (710/ml) when compared with PDA and CMA.

4.5. Biomass production

Use of different culture media had a significant effect (P≤0.05) on biomass production of *P. capsici.* The highest biomass of the fungus was produced on PDA (3.63mg/100ml) which was 24.34% more than the minimum (2.74mg/100ml) obtained on water agar (Table 1). The former was closely followed by CMA (3.34mg/100ml) which was 17.77% more than that produced on Water agar.

Table 1. Effect of different culture media on radial colony growth, sporangial production and biomass production of P. capsici following 15days of incubation at 25°C

Culture medium	Radial colony growth (cm)	No of sporangium/ml	Biomass mg/yield 100ml
1. Potato Dextrose Agar	3.49 a	1430 b	3.63 a
2. Corn Meal Agar	2.62 c	26,000 a	3.34 b
3. Water Agar	3.21 b	710 c	2.74 c
LSD (0.05)	0.0325	2.178	2.178

Means followed by different letters are significantly different from one another at 5% level of significance

4.6. Spore germination

In general, spore germination time ranged from 18-27h for the three media under study (Figure2). ANOVA revealed significant variations (P≤0.05) among the three media. The minimum time taken for 95% spore germination was on PDA (18h) which was 33.3 % less than the maximum time taken on CMA. This was followed by water agar (23h) which was 14.81% less than CMA. The longest (27h) time taken by *P. capsici* sporangia to complete 95% germination was on CMA.

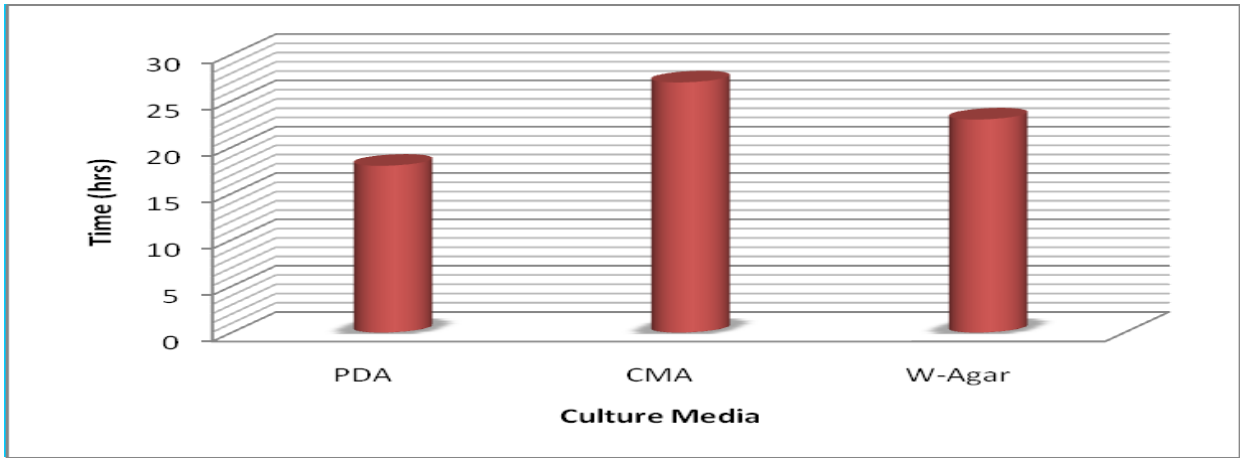

Figure 2. Effect of different culture media on time required to complete 95% spore germination by P.capsici at 25 °C

4.7. Evaluation of phytobiocides *in vitro*

The effects of garlic, peppermint, turmeric, cabbage, radish and onion extracts on the mycelial growth of *P. capsici* are given in Table 2 Garlic, cabbage and peppermint extracts inhibited mycelial growth of *P. capsici more* than the others. Garlic extract encouraged the mycelial growth to 1.25cm which was 63.56 % less than that of control. This was followed by cabbage (1.32cm) and peppermint (1.37cm). Conversely, the extracts of radish and onion had a stimulatory effect on radial growth of *P.capsici* which was only 3.21 % and 2.62 % less than the control, respectively.

Table 2. Effect of various phytobiocides on control of P. capsici in vitro.

Plant Material	Mycelial growth (cm)	Decrease(%) than control
1. Garlic	1.25 f	63.56
2. Cabbage	1.32 e	61.52
3. Peppermint	1.37 d	60.06
4. Onion	9993.34 b	2.62
5. Radish	3.32 c	3.21
6. Control	3.43 a	_
LSD (0.05) = 2.063		

5. Discussion

PCR is a very useful technique for plant disease diagnosis since it is very exact, robust, rapid and less laborious than conventional diagnostic methods. Attempts were made to detect the pathogen in symptomatic and non

symptomatic plant samples of chilli. Strong amplification product of 595bp was obtained from infected symptomatic plant tissues (root) without amplification of any plant DNA exhibiting specificity of the primers. Amplification from apparently healthy, yet diseased plant tissues showed that primer pair can detect sufficiently low levels of DNA present in tissues with latent infection. The ability of the method to amplify DNA from infected yet symptomless plants suggests that it could be a useful method to detect the fungus in the field, even before the onset of symptoms which could thus help in timely management strategies. The tool can possibly be extended in detecting the pathogen in environmental samples such as irrigation water as well as soil to make useful recommendations to the growers.

Three different culture media were tested for different growth parameters. The culture media were inconsistent with respect to various parameters of fungal growth. Potato Dextrose Agar was better in terms of supporting mycelial growth *P.capsici* as compared to other media. A thick cottony off white mycelial growth was observed on PDA while growth on Water Agar and Corn Meal Agar was scant. It was observed that PDA, due to abundant starch content and its rich carbon source supplemented vegetative growth of *P.capsici* thereby resulting in massive mycelial growth. The reason for comparatively less growth on corn meal agar, despite its nutritive value, could be that it is deficient in starch compared to PDA. Conversely, sporangial production was more on corn meal agar than on the other two media which is in line with previous studies (Mina and Cohen, 1972; Khalil and Alsanius, 2009).

Results of spore germination were in contrast with sporangial production experiment. Maximum number of sporangia was produced on CMA mainly due to the fact that it is a rich source of nitrogen and amino acids which are essentially responsible for sporangial production (Singh, 1973). Relatively small number of sporangia germinated on WA and PDA. It is thus suggested that CMA medium is comparatively better for production of reproductive structures such as sporangia whereas PDA can be regarded good for vegetative growth (mycelia). On the other hand, WA was mainly used by the fungus for survival under nutritional stress As regards the 95% sporangial germination time on the three culture media; the minimum time taken for 95% spore germination was on PDA (18h). This was followed by water agar (23h). The longest (27h) time taken by *P. capsici* sporangia to complete 95% germination was on CMA. . Further studies are needed to substantiate these results.

The results of *in vitro* studies on control of *P. capsici* revealed that some antifungal substances are present in garlic, cabbage and peppermint extracts which may possibly be deficient in onion. Garlic is rich in Diallyl disulphide and Allycin in its aromatic oils, which have strong antimicrobial effects (Akg, 1993; Ashurst, 1995; Schwartz and Mohan, 1995), which could have contributed to its ability to restrict mycelial growth of *P. capsici.*

Similarly, biocidal compounds, mainly isothiocyanates, are released by cruciferous plants during the enzymatic degradation of the glucosinolates present in the plant cells. *Brassica juncea* is rich in isothiocyanates due to which it has highest fungicidal activity among all cruciferous plants (Smolinska and Horbowicz, 1999). Likewise peppermint is rich in menthol, menthone, and methyl ester which might inhibit the mycelial growth of *P. capsici* (Heath and Reineccius, 1986) as exhibited by the present studies.

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