# Studies on Penicillium fruit rot (*Penicillium citrinum*) of citrus (*Citrus aurantifolia* Swingle) and its management

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**IN**

**PLANT PATHOLOGY**

**BY**

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# Studies on Penicillium fruit rot (*Penicillium citrinum*) of citrus (*Citrus aurantifolia* Swingle) and its management

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## ABSTRACT

Citrus (*Citrus aurantifolia* Swingle) is the most important fruit crop, belongs to the family *Rutaceae* and sub-family *Aurantioidea.* The important commercial citrus fruits cultivated in India are the Mandarin orange (*Citrus reticulata* Blanco) followed by Sweet orange (*Citrus sinensis* Osbeck.), Acid lime (*Citrus aurantifolia* Swingle) and Lemon (*Citrus limon* Burn.). In India, mandarin constitutes about 41, sweet oranges 23 and acid lime and lemon about 23 per cent of total citrus produced. Lime is a potential source of vitamin-C and it also extensively used for medicine and culinary purposes. In India, citrus fruits rank third in area and production after mango and banana with an estimated production of 96,38,000 MT with an area of 9.87 lakh hectares contributing 13.5 per cent of total fruit production. Gujarat state at present has 37,100 ha. area under citrus (*Citrus aurantifolia* Swingle) crop producing 3.85 lakh tonns fruits with productivity of 10.4 tonns/ha.

Diseased citrus fruits showing typical symptoms of Penicillium rot were collected from Sardar Patel vegetable market, Anand and brought to the laboratory for isolation of the pathogen. The infected fruit exhibit water soaked, light brown, soft areas with olive green spore mass. The Koch's postulates of isolated pathogen were proved on healthy matured citrus fruits following wound inoculation method. The pathogen was reisolated from the artificially inoculated fruits on PDA. The pure culture obtained was sent for identification to Indian Type Culture Collection (I.T.C.C.), I.A.R.I., New Delhi – 110 012 and was identified as *Penicillium citrinum* (ID. No: 8298.11).

The morphological studies revealed that the pathogen (*Penicillium citrinum*) produced bluish green mycelial growth with abundant sporulation on PDA. The conidiophores are branched twice or thrice, elongate, bearing long tangled chains of conidia.

The weekly survey carried out from first week of October to fourth week of March, 2010-11 revealed that the incidence of Penicillium rot was predominant (6.48 & 5.95 %) at both the markets, respectively.

The activities of polygalaturonase and polyme thylgalacturonase enzymes were studied on fruits inoculated with *P. citrinum* along with healthy fruit. The PG activity was higher in fruits inoculated with *P. citrinum* than healthy fruits, while no

significant difference was observed in PMG activity between inoculated and healthy fruit peel.

DNA analysis of five isolates with 15 primers showed amplification of total 160 bands, with a range of 16 to 7 bands per primer. Overall, *Penicillium* isolates exhibited a moderate level of genetic diversity. The maximum 16 bands were generated by a primer 0PA- 12.

Clustering pattern of dendrogram generated by using the pooled molecular data of 14 RAPD loci indicated that two clusters namely 1 and 2 were formed. Cluster 1 includes four isolates *viz.*, isolate AND, ABD, DKR and NAV. While cluster 2 includes single isolate i.e. VAD. The similarity coefficient ranged from 0.42 to 0.53 with all 15 primers. Among the 15 primers the primer viz., OPA-13, OPA-10, OPE-17 and OPE-18 showed 100 per cent polymorphism. These primers can be utilized further to ascertain the variability among the isolates of *P. citrinum.*

Among the fungicides screened *in vitro*, complete mycelial growth inhibition of *P. citrinum* was observed in azoxystrobin and azoxystrobin 18.2% + difenconazole. (250 and 500 ppm), mancozeb (1000 and 2000 ppm), hexaconazole (5%) + captan (70%), carbendazim (12%) + mancozeb (63%), cymoxanil (8%) + mancozeb (64%), carbendazim at both the concentrations (500 & 1000 ppm). The lowest Penicillium rot severity was

recorded in fruits treated with mancozeb at 2000 ppm followed by carbendazim (1000 ppm) both in pre (4.16 %) and post-inoculation (4.99 %) treatments, respectively at 8 days after inocubation.

Complete mycelial growth inhibition was recorded in tulsi, garlic, and henna extract. Further tulsi leaf extract (10%) found most effective in reducing the Penicillium fruit rot severity both in pre- (3.00 %) and post-inoculation (4.00 %) treatments at 8 days after inoculation.

*Trichoderma harzianum* found most efficient antagonist in inhibiting the mycelial growth of *P. citrinum* (77.41 %) *in vitro.* It was also found most effective in reducing the Penicillium rot severity in pre inoculation (10.00 %) treatment at 8 days after inoculation.

The fruits exposed to hot water treatment at all five treatments showed 100 per cent reduction in Penicillium rot incidence over control up to ten days of storage at 25±1°C without any changes in natural fruit colour. The protein pattern of citrus fruit peels treated with HWT showed the presence of 103 KDa of molecular weight of proteins but these bands were absent in healthy and untreated and inoculated protein samples. The profile results showed the bands ranging from 30-35 number with protein molecular weight ranging from 15 KDa to 195 KDa.

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# *CERTIFICATE*

This is to certify that the thesis entitled **"STUDIES ON PENICILLIUM FRUIT ROT (*Penicillium citrinum*) OF CITRUS (*Citrus aurantifolia* Swingle) AND ITS MANAGEMENT" submitted by SWETHA REDDY (Reg. No. 04-1358-2010)** in partial fulfilment of the requirements for the award of the degree of **MASTER OF SCIENCE (AGRICULTURE)** in **PLANT PATHOLOGY** of the Anand Agricultural University, Anand is a record of bonafide research work carried out by her under my personal guidance and supervision and the thesis has not previously formed the basis for the award of any degree, diploma or other similar title.

**Place : Anand** **(R.K. Patil)**
**Date : /08/ 2012** **Major Guide**

# DECLARATION

This is to declare that the whole of the research work reported here in the thesis is for the partial fulfilment of the requirements for the Degree of **MASTER OF SCIENCE (AGRICULTURE)** in **PLANT PATHOLOGY** by the undersigned is the result of investigations done by me under the guidance and supervision of **Dr. R. K. Patil**, Professor, Department of Plant Pathology, B. A. College of Agriculture, Anand Agricultural University, Anand and no part of the work has been submitted for any other degree so far.

**Place**: Anand

**Date**: /08/2012 (**Swetha Reddy)**

**Countersigned by**

(**R. K. Patil)**

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***Place : Anand***

***Date :*** */08/2012* *(Swetha Reddy)*

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# *Abbreviations and Acronyms*

| | |
|---|---|
| % | Per cent |
| @ | At the rate of |
| µ | Micron |
| µg | Microgram |
| µl | Microliter |
| µm | Micrometer |
| $^0$C | Degree Celsius |
| AAU | Anand Agricultural University |
| Anon. | Anonymous/ Unacknowledged |
| APS | Ammonium per sulphate |
| B.A. | Bansilal Amritlal |
| BPB | Bromophenol blue |
| C.D. | Critical difference |
| C.V. | Coefficient of variation |
| C: I | Chloroform: isoamyl alcohol |
| Cfu | Colony forming unit |
| CTAB | Cetyl-trimethyl-ammonium-bromide |
| DNA | Deoxyribo Nucleic Acid |
| dNTP | Deoxynucleotide 5' triphosphate |
| e.g | For example |
| EC | Emulsifiable concentrate |
| EDTA | Ethyl-diamine-tetra acetic acid |
| *et al.* | *et allii* ; and coworkers |
| etc. | Et cetera., pretty much the alike |
| Fig. | Figure |
| G | Gram |
| G.A.U. | Gujarat Agricultural University |
| Gly | Glycine |
| H | hour |
| $H_2SO_4$ | Sulphuric acid |
| ha. | Hectare |
| HCL | Hydrochloric acid |
| Hrs | hours |
| I.A.R.I. | Indian Agricultural Research Institute |
| i.e. | That is |
| I.T.C.C. | Indian Type Culture Collection |
| *In vitro* | Laboratory condition |
| *In vivo* | Field condition |
| Kb | Kilo base pairs |
| Kda | Kilo Dalton |
| Kg/cm$^2$ | Kilogram per centimeter square |
| L | litre |
| M | meter |
| M | Molar |
| M ha | Million hectare |
| Mg | Milligram |
| Min | Minutes |
| Ml | Milliliter |

| | |
|---|---|
| mM | milli Molar |
| Mm | Millimeter |
| MT | Metric tone |
| N | Normal |
| $N_2$ | Liquid nitrogen |
| NaCl | Sodium chloride |
| Ng | nanogram |
| Nm | nanometer |
| O.D. | Optical density |
| PAGE | Poly acrylamide gel electrophoresis |
| PCR | Polymerase chain Reaction |
| PDA | Potato Dextrose Agar |
| PGI | Per cent Growth Inhibition |
| pH | Potential of hydrogen ion |
| Ppm | Parts per million |
| PVP | Polyvinyl-pyrrolidone |
| RAPD | Random amplification of polymorphic DNA |
| RH | Relative humidity |
| RNA | Ribose nucleic acid |
| rpm | Revolution per minute/ rotation per minute |
| RT | Room temperature |
| S.Em | Standard Error of mean |
| SDS | Sodium dodecyl sulphate |
| Sec | Second |
| spp. | Species |
| Sr. No. | Serial Number |
| TBE | Tris- borate-EDTA-buffer |
| TE | Tris-EDTA-buffer |
| TEMED | N,N,N',N' – tetra methyl ethylene diamine |
| Tr. No. | Treatment Number |
| UPGMA | Unweighted pair group method with arithemetic mean |
| v/v | Volume by volume |
| viz., | Namely |
| w/v | Weight by volume |
| WP | Wettable powder |
| β-ME | β-mercaptoethanol |
| UV | Ultraviolet |

# I. INTRODUCTION

Citrus (*Citrus aurantifolia* Swingle) is the most important fruit crop, belongs to the family *Rutaceae* and sub family *Aurantioidea.* It is said to be indigenous to South East China (Singh and Jain, 2004). Citrus is cultivated in more than 100 countries covering all six continents and it is often regarded as golden fruit or queen of all fruits (Nito, 1996). It is also known as Kagzi-lime, Acid lime, Sour lime, Mexican lime and in Hindi as “Neebu” and in Gujarati as “Leembu”.

The important commercial citrus fruits cultivated in India are the Mandarin orange (*Citrus reticulata* Blanco) followed by Sweet orange (*Citrus sinensis* Osbeck.), Acid lime (*Citrus aurantifolia* Swingle) and Lemon (*Citrus limon* Burn.). In India, mandarin constitutes about 41, sweet oranges 23 and acid lime and lemon about 23 per cent of total citrus produced (Ghosh, 1997).

Acid lime plant is a perennial profusely branched thorny shrub or small tree which bears more or less round oval, smooth fruits having thin rind attached lightly. Mature fruits of lime are light yellow in colour (Singh, 1995). Lime is a potential source of vitamin-C and it also extensively used in medicine and culinary purposes (Singh, 1995). The most popular citrus products prepared in India are squash, juice, cordinal,

marmalade, pickles as well as essential oils from citrus peel is used for flavoring and perfumery trade, also used in manufacturing of soap and resins etc. (Randhawa and Srivastava, 1986).

In India, citrus fruits rank third in area and production after mango and banana with an estimated production of 96,38,000 MT with an area of 9.87 lakh ha. (Anon, 2010a) contributing 13.5 per cent of total fruit production in India. Commercial cultivation of citrus is observed mainly in Maharashtra, Andhra Pradesh, Karnataka, Gujarat, Punjab, Orissa and Madya Pradesh. Gujarat state at present has 37,100 ha. area under citrus (*Citrus aurantifolia* Swingle) crop producing 3.85 lakh tonns fruits with productivity of 10.4 tonns/ha. (Anon., 2010a).

Lime fruits and many other fruits are perishable in nature. During peak season, there is a glut in the market compelling the farmers to sell their produce at throw-away prices and hence the fruits are transported to long distance either for storage or for selling to fetch more prices. The lime fruits during harvest, transit and storage subjected to minor injuries / wounds resulting in early invasion by several fungi to cause the post-harvest fruit rots. The retailers as well as wholesalers have to bear losses from these rots resulting into lower prices.

The fruit rots in citrus (*Citrus aurantifolia* Swingle) caused by various species of fungi includes Penicillium, Alternaria, Aspergillus, Colletotrichum, Botryodiplodia and Phomopsis are the most predominant as they affect the fruit quantity, quality and ultimately reduces the marketable value (Bhardwaj and Sharma, 1999). Naqvi and Das (1994) surveyed the several districts of maharastra and found that 43 and 47 per cent losses of mandarins was observed in truck and train transport, respectively. The post harvest diseases i.e. *Lasiodiplodia theobromea, Colletotrichum gleosporioides, Alternaia citri* and *Phomopsis citri* contributed 21-26 per cent losses followed by *Geotrichum candidum* (13-15%), *Penicillium digitatum* and *P. italicum* (1-4%). Kaur and Verma (2002) surveyed the prevalence of post harvest rots of kinnow in Punjab, where the incidence of *Penicillium digitatum* (58.33%) was identified as the major post harvest disease incurring 58.33 per cent yield loss followed by *Alternaria citri* (33.3%), *Penicillium italicum* (26.7%), *Aspergillus niger* (20%), *Phytopthora* spp. (13.3%). Singh and Thakur (2003) reported that the *Penicillium digitatum* was most predominant post harvest fungal pathogen followed by *Alternaria citri* and *P. italicum* during the storage of kinnow fruits. Singh and Jain (2004) recorded the major mycoflora causing losses of kinnow mandarins i.e. *Alternaia alternata* (81.1%), *Penicillium digitatum* and

*Penicillium italicum* (64-70%) in long distance marketing. Verma (2009) observed major fruit spoilage of Kinnow mandarins from November to April due to *Penicillium digitatum* (5.18-8.85%) and *P. italicum* (2.59-4.82%) with peak losses in January (8.25%), February (8.85%) and March (4.43%), respectively.

Realizing the gaps in our knowledge and management of the post-harvest disease; it was felt worthwhile to carry out the investigations on Penicillium rot of citrus and its management under middle Gujarat conditions with following aspects.

1. Isolation, Purification, Pathogenecity and Identification of the pathogen causing Penicillium rot of citrus.
2. Survey of post-harvest diseases of citrus at Sardar Patel Market, Station road and Goya Talav Market at Anand.
3. Studies on cell wall degrading enzymes.
4. Molecular variability among the isolates of *Penicillium* causing mold rot in citrus fruit using RAPD markers.
5. Evaluation of fungicides, phytoextracts and antagonists against Penicillium rot of citrus *in vitro* and *in vivo.*
6. Effect of Hot Water Treatment on Penicillium fruit rot of citrus.
7. Protein profile (SDS-PAGE) analysis of Hot Water treated inoculated and healthy citrus fruit peel.

# II. REVIEW OF LITERATURE

## 2.1 Isolation, Purification, Pathogenecity and Identification of pathogen causing Penicillium rot.

Verma (2008) isolated seven fruit rotting pathogens viz., *Penicillium digitatum, P. italicum, P. chrysogenum, Aspergillus niger, Alternaria alternate, Fusarium moniliforme* and *Botryodiplodia theobromae* from infected fruits of citrus by tissue method.

Oliveri1 *et al.* (2007) proved the pathogenicity of *Penicillium* strains on citrus fruits by wounding method. Each fruit was wounded with a steel sterile needle at four locations in the equatorial region. The wounded fruits were inoculated with 10 µl of conidial suspension ($10^6$ conidia $ml^{-1}$) from the 7 day-old culture.

Smilanick *et al.* (2005) isolated *Penicillium digitatum* from infected lemon fruits and proved the pathogenicity by making wound with a steel rod and followed by dipping in fungal spore suspension.

Mishra (1988) isolated *Penicillium* spp. from stored rotted fruits of aonla using standard blotter and agar plate methods. Gupta and Chauhan (1985) isolated two *Penicillium*

spp. from rotted fruits of aonla and proved the pathogenicity of these *Penicillium* spp.

## 2.2 Survey of post harvest diseases of citrus fruit

Naqvi and Dass (1994) noted 43 and 47 per cent losses in mandarin due to post harvest diseases when fruits were transported in truck and train, respectively. Among them, *Botryodiplodia* sp., *Colletotrichum* sp., *Alternaria citri* and *Phomopsis citri* contributed 21-26 per cent losses, while considerable amount of losses were also reported due to *Geotrichum candidum* (13-15%), *Penicillium digitatum* and *Penicillium italicum* (1-4%).

Kaur and Verma (2002) surveyed the markets of Punjab from mid January to second week of March. They recorded highest incidence of green mould rot (*Peenicillium digitatum*) (58.33 %), followed by *Alternaria citri* (33.3 %), *P. italicum* (26.7 %), *Aspergillus niger* (20 %) and *phytopthora* sp. (13.3%) infecting kinnow. Dinesh and Thakur (2003) observed highest incidence of *Penicillium digitatum* followed by *Alternaia citr*i and *P. italcum* both in ambient and cold storage conditions in kinnow fruit.

Jain and Singh (2004) noted highest incidence of Alternaria fruit rot caused due to *Alternaria alternata* (81.1 %) in kinnow followed by *Penicillium digitatum* and *P. italicum* (64-70 %).

Verma (2009) surveyed the post-harvest diseases of kinnow mandarin at wholesale fruit mandi and retail fruit markets of Jammu. He reported the major fruit spoilage during November to April due to green and blue mold rots caused by *P. digitatum* (5.18-8.85 %) and *P. italicum* (2.59-4.82 %), respectively.

Verma and Tikoo (2004) carried out weekly survey of wholesale fruit mandi, Narwal as well as various retail markets of Jammu round the year and reported that post-harvest losses due to various pathogens in acid lime ranged from 5-54 per cent. Highest losses were caused by *A. niger* (10.89 %) in August followed by green mold (*P. digitatum*) (2.75 %) and blue mold (*P. italicum*) (1.89 %) in November.

Reddy *et al.* (2008) reported highest postharvest fungal spoilage in acid lime due to *Penicillium digitatum, Aspergillus niger* and *Geotrichum candidum.*

## 2.3 Studies on cell wall degrading enzymes

Barmore and Brown (1979) studied the role of pectolytic enzymes and galacturonic acid in citrus fruits decay caused by *P. digitatum* and reported that there was reduction in tissue strength, which was apparent with three constituents i.e. galacturonic acid, PME (Pectin methyl esterase) and exo-PG (exo-Polygalacturunase) causing specific cellular changes and collectively resulting in weakening of the cell wall.

Hershenhorn *et al.* (1990) studied the association of Polygalacturonases in relation to infection by *P. italicum* in Valencia orange and concluded that high exo-PG activity in infected tissues may be responsible for the excessive accumulation of D-galacturonic acid which occurs during blue mold infection.

Brown and Barmore (1983) studied the resistance of healed exocarp in penetration by *P. digitatum* and concluded that the accumulation of phenolic and lignin like materials in cell wall of injured exocarp may inhibit fungal growth and interfere with enzymatic degradation or lignify penetrating hyphae.

## 2.4 Molecular variability among the isolates of *Penicillium* causing mold rot in citrus fruit using RAPD markers

Schmidt *et al.* (2006) collected *Penicillium digitatum* isolates from infected lemons and oranges from various geographically diverse locations of California. Thirty-five isolates collected from commercial groves and residential trees which were sensitive to TBZ, while 19 of 74 isolates collected from 10 packing houses were found resistant to TBZ. Random amplified polymorphic DNA analysis indicated that the isolates were genetically distinct and differed from each other.

Araújo *et al.* (2007) studied two species of the genus *Penicillium* (*P. expansum* and *P. griseoroseum)* collected from

infected forest seeds from Brazil for molecular and morphological characterization. Total 78 RAPD primers were used, among which 8 showed differences in band patterns with 54 per cent of the amplified polymorphic fragments.

Pianzzola *et al.* (2004) studied the genetic characterization of *Penicillium* isolates associated with blue mold on apple by using RFLP and RAPD primers. The results showed that out of all isolates, 11 were identified as *P. expansum* and 3 as *P. solitum.*

## 2.5 Evaluation of antagonists, phytoextracts and fungicides on Penicillium rot of citrus fruit *in vitro* and *in vivo*

### 2.5.1 Fungicides.

Verma (2008) evaluated six fungicides against *Penicillium digitatum* and *P. italicum* causing green and blue mold rots of mandarin oranges by pre and post inoclution treatments and concluded that thiobendazole (0.05%), benomyl (0.05%) and carbendazim (0.05%) were highly effective in controlling green and blue mold rots of mandarin oranges.

Maharshi *et al.* (2009) screened nine fungicides against *P. italicum in vitro* and *in vivo.* They reported that prochloraz proved significantly superior in inhibition of mycelial growth at lowest concentration (5 ppm).

Singh and Thakur (2005) evaluated efficacy of three fungicides on fruit rot of kinnow fruits caused by *P. italicum* and reported that carbendazim at 1000 ppm conc. found most effective followed by mancozeb (1000 ppm) and copper oxychloride (1000 ppm) both *in vitro* and *in vivo*.

Kanetis *et al.* (2007) studied the effectiveness of fungicides (azoxystrobin, fludioxonil & pyrimethanil) against citrus green mold *in vivo.* They reported that the mixture of imazalil (500 mg/L) with pyrimethanil (500 mg/L) found most effective in controlling decay due to green mold rot (*P. digitatum*).

Timmer (1973) observed that pre harvest application (13 & 60 days) of benomyl (100 & 250 ppm) was most effective against the Penicillium rot in oranges. Javed *et al.* (1995) reported that thiobendazole at 0.4 per cent was most effective in inhibiting the mycelial growth of *Aspergillus niger, Alternaria citri, Penicillium digitatum* and *P.italicum* infecting citrus fruits. Kaul and Sharma (1988) advocated that pre harvest spray of carbendazim (750µg/ml) found most effective in preventing blue mold rot *(Penicillium expansum)* of apple. Raju and Naik (2006) tested the efficacy of various systemic pre harvest fungicidal sprays. Application of SAFF, benomyl and tridemifon (0.1 & 0.15%) gave complete inhibition of mycelial growth (*Penicillium digitatum*) followed by carbendazim (81.78 & 100%) in onion.

### 2.5.2 Phytoextracts

Sharma *et al.* (2009) studied the fungitoxic properties of some indigenous aromatic plant extracts on blue mold rot of kinnow fruits caused by *P. italicum* and concluded that eucalyptus, garlic and henna each at 15 per cent conc. showed 80, 60 and 54 per cent mycelial growth inhibition, respectively.

Tripathi and Dubey (2003) evaluated the antifungal activity of leaf extracts of 24 angiospermic plants and bark extracts of *Acacia catechu, A. farnesiana* and *A. nilotica* against blue mold rot (*P. italicum*) of mandarin oranges. They reported that the bark extract of *Acacia farnesiana* in ethyl acetate found highly effective even at much higher dilution.

Raju and Naik (2006) screened the various botanicals against blue mold rot of onion caused by *P. digitatum.* Nimbicidin (5%) and garlic extract (5%) gave 100 and 67.46 per cent mycelial growth inhibition, respectively.

Yigit *et al.* (2000) studied the inhibitory effect of essential oil of black thyme, dill, coriander and rosemary on *P. digitatum* causing post harvest rot in citrus. They reported that the vapour of these oils inhibited mycelial growth of pathogen to 85.8, 82. 8, 80 and 71.4% per cent, respectively.

Nath *et al.* (2012) evaluated the antifungal activity of *Azadirachta indica*, *Ocimum sanctum*, *Aloe barbedensis,*

carbendazim, *A. barbedensis* + *A. indica*, *A. barbedensis* + *O. sanctum*, *A. barbedensis* + carbendazim against *P. bravipectum* infecting khasi mandarin. They found that *O. sanctum* (50%)-treated fruits exhibited minimum decay loss (3.33%) up to a total period of 28 days which was even less than carbendazim (10%).

### 2.5.3 Antagonists

Stockwell *et al.* (2007) applied *Pseudomonas syringae* strains ESC-10 and ESC-11 in packing houses to prevent postharvest fungal diseases during storage, these strains were found effective for the control of postharvest diseases of citrus, stone fruit and potato.

Zhang and Dou (2002) studied two *Bacillus subtilis* strains GB0 3 and GB0 7, to test their biocontrol activity against green mold rot caused by *Penicillium digitatum* on Valencia orange. The strain GB0 7 found most potential to control green mold (72.2 to 100 %).

Godara (1994) found *Trichoderma harzianum* as most potent antagonist in inhibiting the growth of *Penicillium italicum* and *Botryodiplodia theobromae* infecting sweet oranges.

Bhuvaneswari and Rao (2001) reported that *Trichoderma viride* found most effective in controlling Penicillium rot in mango fruits.

Bagwan (2003) reported the potentiality and viability of *Trichoderma* spp. and *Candida* spp. in controlling the green *(P. digitatum)* and blue mold *(P. italicum)* rots of citrus respectively.

Etebarian *et al.* (2005) obtained best biological control of apple blue mould (*Penicillium expansum* & *Penicillium solitum*) with *Pseudomonas fluorescens.*

Chettri *et al.* (2005) reported that the native fluroscent *Pseudomonas* isolates were most efficient in inhibiting the growth of green (*P. digitatum*) and blue mold (*P. italicum*) rots of mandarin oranges.

## 2.6 Effect of Hot Water Treatment on *Penicillium* spp. causing Penicillium mold rot in citrus

Inkha and Boonyakiat (2010) found that the tangarine fruits when dipped in hot water at 50 ± 2°C for 3 min. and 55 ± 2°C for 2 and 3 min. after inoculation with *P. digitatum,* remarkably delayed the onset of disease initiation and reduce the number of infected fruits with low disease severity.

Palou *et al.* (2001) studied the effect of hot water treatment on oranges at temperatures 35, 45, 48, 50, 53, 55, 57, 60, 65, and 75 (± 1°C) to control the blue mold rot pathogen (*P. italicum*). They reported that the hot water treatment at 55°C controlled the *P. italicum* effectively after 7 days of storage at 20 °C.

Smoot and Melvin (1963) reported that the immersion of oranges in hot water at 53ºC controlled green mold rot (*P. digitatum*) effectively.

Houck (1967) found that the green mold rot (*P. digitatum*) of Furaka lemons was effectively controlled at 52 ºC.

## 2.7 Effect of Hot Water Treatment on protein patterns (SDS-PAGE) of citrus fruit peel

Inka and Boonyakiat (2010) investigated the effect of hot water treatment in enhancing the host resistance to green mold rot caused by *Penicillium digitatum.* Results showed an increase in chitinase and beta-1, 3-glucanase activities in flavedo tissue of treated fruits, protein patterns of treated fruit peel which have 112.20 and 100.00 kDa proteins on fifth day of storage.

Barraclough *et al.* (2004) optimised the methods for the extraction and two-dimensional electrophoresis (2-DE) of fruit proteins for a range of fruit tissues including low protein sources (apple and peach flesh), high lipid material (avocado fruit flesh) and high acidity lemon tissues.

Pavaoncello *et al.* (2001) showed that hot water brushing treatment at 62 ºC for 20 sec. against *Penicillium digitatum* in grape fruit (Star Ruby) induced the accumulation of

the 21, 22 and 25 kDa chitinase protein and of 38 and 43 kDa β-1-3 glucanase proteins.

Mccollum *et al.* (1997) reported that the 22 kDa protein is one of the chitinase isoform that is normally abundant in heat stressed grape fruit.

# III. MATERIALS AND METHODS

Present investigation on "Studies on Penicillium fruit rot (*Penicillium citrinum*) of Citrus (*Citrus aurantifolia* Swingle) and its management" was carried out in the Department of Plant Pathology at Bansilal Amritlal College of Agriculture, Anand Agricultural University, Anand during the year 2011-12. The materials used and methods adopted during the studies are described here in this chapter.

## 3.1 General

### 3.1.1 Location

Anand Agricultural University, Anand, where the present investigation was under taken is situated on $22^0$-35' North latitude and $72^0$-55' East longitude and has an elevation of 45 meters above the mean sea level.

### 3.1.2 Climate

Monsoon is warm and moderately humid. It commences by the middle of June and ends in middle of September. An average rainfall of the tract is about 851.68 mm. Monsoon in this area is often erratic and uncertain, both in respect of total rainfall and its distribution. Winter is fairly cool and dry, while summer is quite hot and dry.

### 3.1.3 Laboratory procedures

#### 3.1.3.1 Glassware cleaning

Standard glasswares were used during all the laboratory experimentation. The glasswares were dipped in cleaning solution – chromic acid (60 g of potassium dichromate dissolved in 300 ml of distilled cooled water, then after 400 ml of concentrated sulphuric acid was added in it with constant stirring) for 24 hours. Afterwards the glasswares were cleaned with detergent powder, followed by washing in running tap water and finally rinsed with distilled sterile water and dried before use.

#### 3.1.3.2 Sterilization

The cleaned glasswares were sterilized at $180^0$ C for 2 hours in hot air oven. Media and water were sterilized in an autoclave at $121^0$ C at 1.1kg/$cm^2$ pressure for 20 min.

### 3.1.4 Experimental materials

Fresh, healthy and matured citrus fruits were collected from Sardar Patel vegetable market, station road, Anand.

### 3.1.5 Selection of variety

The citrus variety **'kagzi lime'** was selected for studies, as it is better in terms of yield, size, juice and citric acid content and preferred by the growers and consumers.

## 3.2 Collection, isolation, purification, pathogenicity and identification of pathogen

### 3.2.1 Collection, isolation and purification

Fresh, naturally infected diseased citrus fruits showing typical symptoms of Penicillium fruit rot were collected from the Sardar Patel vegetable market, Anand and brought to the laboratory for isolation of the pathogen. The fruits were washed in tap water and after drying small pieces of diseased tissues along with adjoining healthy tissues were cut with sterilized blade and surface sterilized by dipping in 0.1 per cent NaOCl solution for one minute followed by three successive washings with distilled sterile water and these pieces were placed on Potato Dextrose Agar (PDA) medium (20 ml) poured in Petri plates under aseptic condition. The inoculated plates were incubated for growth of the pathogen at 25 ± $1^0$ C in BOD incubator for seven days. The pure mycelial growth was subcultured and maintained on PDA slants. From seven days old pure culture spore suspension was made following serial dilution in distilled sterile water so as to get two to three spores per drop of suspension. One ml. of such diluted suspension was poured aseptically on the solidified sterilized molten water agar medium. Petri plates were tilted in different direction to get uniform spread of spore suspension. Petri plates were examined under the stereoscopic binocular research microscope. Well-

isolated single spores were marked and then transferred on PDA slants separately under aseptic conditions and incubated at 25 ± $1^0$ C in BOD incubator for seven days. The pure culture thus obtained was maintained by periodical transfer on PDA slants throughout the investigations.

### 3.2.2 Pathogenicity

Matured healthy citrus fruits of 'kagzi lime' cultivar were collected from Sardar Patel vegetable market, Anand. The fruits were thoroughly washed in tap water, followed by washing with distilled sterile water then surface sterilized by dipping in 0.1 per cent NaOCl solution for two minutes followed by three successive washings with sterile distilled water and then inoculated with isolated pathogen. The spores, harvested from seven days old culture, were mixed in 200 ml of distilled sterile water with tween 80 (0.05% wt/vol). The fruits were wounded at equator region with sterilized pins which were fixed on cork. The fruits were then inoculated with 25µl spore suspension ($10^6$ spores/ml) using a micropipette on site of wounds. The inoculated and uninoculated (dipped in distilled sterile water) fruits were placed separately in sterilized plastic tray containing moist absorbent cotton at the bottom and covered with sterilized polythene sheet. The packed fruits were kept at 25 ± $1^0$ C in BOD incubator for seven days. The fruits

were examined daily for symptom expression. The fruits showing typical Penicillium rot symptoms were used for reisolation of the pathogen. The cultural and morphological characters of reisolated pathogen were compared with previously isolated pathogen from naturally infected diseased citrus fruits.

### 3.2.3 Identification

Identification of the pathogen was carried out by studying the cultural and morphological characters. The microphotographs of mycelium and spore structure were taken with the help of digital camera. The pure culture was sent to Indian Type Culture Collection (I.T.C.C.), Division of Mycology and Plant Pathology, I.A.R.I., New Delhi – 110 012 for identification.

## 3.3 Survey of fruit rots of citrus at Sardar Patel vegetable market, Station Road and Goya Talav market at Anand

Weekly survey of fruit rots of citrus was carried out at Sardar Patel vegetable market, Station Road and Goya Talav market at Anand to study the incidence of fungal rots, from first week of October to fourth week of March during 2011-12. Five samples were selected randomly from retailers each containing 100 fruits from both the locations and were examined for the incidence of fungal rots caused due to different pathogens. The

per cent fruit rot incidence was calculated by following standard formula:

$$\text{Disease incidence (\%)} = \frac{\text{Number of infected fruits}}{\text{Total number of fruits examined}} \text{ X } 100$$

### 3.4 Studies on cell wall degrading enzymes

Matured healthy fruits were surface sterilized and separately inoculated with *Penicillium citrinum* by wound inoculation method. The inoculated fruits were incubated at ambient temperature. On 1st, 2nd, 3rd and 4th day, extracts from fruits were obtained according to the procedure described by Bell *et al.* (1955).

Five gram of the rotted and healthy fruit tissues were macerated separately with the help of a pestle and mortar in distilled water (15 ml) and 0.5 N NaCl (15 ml). The ground tissues extract were strained through several layers of cheese cloth. The filtrates were separately centrifuged at 4000 rpm for 20 min. The supernatant was used for cell wall degrading enzyme activity.

The compositions of the reaction mixtures for 2 ml of enzyme sample for the different enzymes are as follows:

**Polymethylgalacturonase (PMG)**

Five ml of one per cent pectin dissolved in buffer solution (pH 5.0), 1.8 ml of 0.1 M phosphate citrate buffer (pH 5.0) and 1.5 ml of distilled water.

**Polygalacturonase (PG)**

Five ml of one per cent sodium polypectate dissolved in buffer solution (pH 5.0), 1.8 ml of 0.1 M phosphate citrate buffer (pH 5.0) and 1.5 ml of distilled water.

The enzyme activity was assessed by determining the loss in viscosity of the reaction mixture immediately at intervals of 10, 30 and 120 minutes at $30^0$C on $1^{st,}$ $2^{nd}$, $3^{rd}$ and $4^{th}$ day. Each treatment was replicated for four times.

The per cent enzyme activity was calculated by the following formula:

$$\frac{Vo - Vt}{Vo - Vw} \quad X \quad 100$$

Where, Vo = The flow time at 0 min.

Vt = The flow time after 10/30/120 min.

Vw = The flow time of distilled water.

## 3.5 Molecular Characterization

Molecular characterization of isolates in respect to RAPD was done according to standard protocol for fungus. Five isolates of *P. citrinum* collected from middle and south Gujarat *viz.* Anand (AND), Vadodara (VDA), Ahmedabad (ABD), Dakor (DKR) and Navsari (NAV). DNA extraction of the five isolates was done by CTAB method with slight modification (Niu *et al.,* 2008) and DNA was amplified with RAPD-PCR technique using random primers.

### 3.5.1.1 Genomic DNA Extraction from Mycelia

### 3.5.1.2 Chemicals used for DNA isolation:

**1)** DNA Extraction Buffer

10 % CTAB

1 M Tris-HCl (pH 8.0)

0.5 M EDTA (pH 8.0)

5M NaCl

Double distilled water

β- mercaptoethanol

**2)** Chloroform : iso-amyl alcohol (C:I) solution in the ratio of 24:1

**3)** DNase-free RNase A (10 mg/ml)

**4)** 70 % Ethanol (Chilled, Absolute)

**5)** TE buffer (1M Tris-HCl (pH 8.0) and 0.5M EDTA (pH 8.0)

### 3.5.2.1 Protocol for isolation of fungal genomic DNA

The culture of *P. citrinum* was grown in Potato dextrose broth medium in automatic shaking incubator at 25±1°C for 7days. Mycelia was collected by filteration through Whatman filterpaper No. 42 and then extensively washed with sterilized water. The mycelium was blotted dry between the layers of tissue and immediately frozen in liquid nitrogen in foil packets. The tissues were gently broken into fine pieces by crushing the foil envelops with a pestle and mortar. Care was taken not to allow the tissue to thaw as this causes lyses and the release of endogenous nuclease.

### 3.5.2.2 Protocol for DNA Extraction

1. Pre-cooled a pestle and mortar at 4 $^0$C then grounded 300 mg mycelium to a fine powder in liquid nitrogen and transferred to a plastic sterile tube by ensuring that the tissue did not thaw.
2. Added to it 10 ml prethawed isolation buffer to it and incubated for 60 minutes with occasional stirring.
3. Extracted for 10 minutes with equal volume of chloroform: isoamylalcohal (24:1).
4. Centrifuged at 10,000 rpm for 20 minutes at room temperature (24$^0$C).
5. Separated the aqueous phase and transfer to a fresh tube.
6. To this aqueous phase added 0.6 volume of ice cold isopropanol and 0.1 volume of sodium acetate, and incubate at -20 $^0$C for 30 minute.
7. Centrifuged the supernatant 10,000 rpm for 10 minutes at 4 $^0$C, discard the aqueous phase.
8. Again washed the DNA pellet obtained with 70% ethanol (5ml).
9. Centrifuged at 10,000 rpm for 10 minutes at 4 $^0$C and then discarded the aqueous phase.
10. DNA pellet dried and then dissolved in 200µ l of TE buffer.

11. Then RNase treatment was given to the samples where in 6 µl of RNase was added and samples were kept at 37 °C in hot water bath for 1 hr. and latter at 65° C for 10 min.

### 3.5.2.3 Spectrophotometric Analysis (Nanodrop):

To check the quality and quantity of isolated genomic DNA in relation to protein and RNA, spectroscopic analysis on nanodrop was carried out. The data were analysed by using the software N.D. (V.3.3.0). One µl DNA was loaded into the well of nanodrop spectrophotometer and the concentration of DNA and absorbance at 260 nm /280 nm was measured. Quantification and purity of DNA was checked on 0.8% agarose gel at voltage of 60V/cm by using 1X TBE buffer and ethidium bromide (4 µl/70 ml) staining. On completion of run, gel was observed under UV light and observations were recorded on Alpha Ease FC4.0.0 gel documentation system.

### 3.5.2.4 Random Amplification of Polymorphic DNA (RAPD) analysis:

A total of 40 decamer primer belonging to OPA and OPE series were screened for RAPD analysis. Of these, 15 primers were selected based on repeatability. The list of RAPD primers which was used for the analysis of random amplification of polymorphic DNA to study the polymorphism present in the isolates of *P. citrinum* have been mentioned in Table 3.1

**Table 3.1: List of primers used for the RAPD analysis**

| Sr.No. | Primer | Sequence (5'-3') | Bases |
|---|---|---|---|
| 1 | OPA-1 | AAT CGG GCT G | 10 |
| 2 | OPA-5 | GTG ACG TAG G | 10 |
| 3 | OPA-9 | GGG TAA CGC C | 10 |
| 4 | OPA-10 | GTG ATC GCA G | 10 |
| 5 | OPA-11 | CAG CAC CCA C | 10 |
| 6 | OPA-12 | AGG TGA CCG T | 10 |
| 7 | OPA-13 | GTT GCG ATC C | 10 |
| 8 | OPA- 18 | CCA GAT GCA C | 10 |
| 9 | OPE- 3 | GTG ACA TGC C | 10 |
| 10 | OPE-11 | AGA TGC AGC A | 10 |
| 11 | OPE-12 | CCC GAT TCG G | 10 |
| 12 | OPE-14 | TGC GGC TGA G | 10 |
| 13 | OPE-15 | ACG CAC AAC C | 10 |
| 14 | OPE-17 | ACG GCG TAT G | 10 |
| 15 | OPE-18 | AAC GGT GAC C | 10 |
| Total | | | **150** |

### 3.5.2.5 PCR Protocol:

**Preparation of Reaction Mixture:**

The reaction mixture for RAPD analysis was prepared as under:

| | |
|---|---|
| Millipore Sterilized Water | - 17.5 µl |
| Taq Buffer A (10X) | - 2.5 µl |
| dNTPs (2.5mM each) mix | - 0.5 µl |
| Taq polymerase (3U/µl) | - 0.5 µl |
| Primer (10 pmoles/µl) | - 1.0 µl |
| Template DNA 40 ng/µl) | - 3.0 µl |
| Total | - 25 µl |

The master mix was prepared by adding first millipore sterilized water followed by Taq buffer A 2.5 µl, 0.5 µl dNTPs, 0.5 µl Taq polymerase, 1 µl primer and finally 3 µl template DNA. The reagents were mixed thoroughly by slight vortex followed by spinning in micro centrifuge. The tubes were then placed in thermal cycler for cyclic amplification. The conditions for amplification were as follows:

### 3.5.2.6 Programming for PCR:

Step-1: 94° C for 4 min. (Initial denaturation)

Step-2: 94° C for 1 min. (Denaturation after every cycle)

Step-3: 34° C for 2 min. (Primer annealing)

Step-4: 72° C for 2 min. (Extension of annealed primer)

Step-5: 72° C for 10 min. (Final Extension)

Step 2, 3 and 4 comprised of one cycle and the total reaction was carried out for 40 cycles.

PCR products were run on electrophoresis with known molecular marker (mol wt. 100 bp) in 1.5 % Agarose gel.

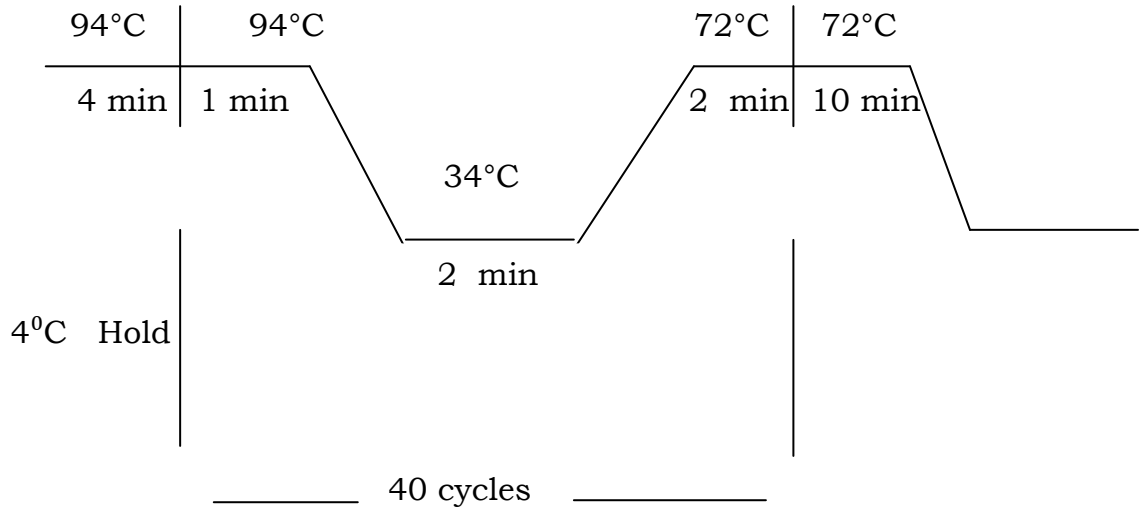


**Figure 3.1: Steps in PCR amplification**

### 3.5.2.7 Data scoring:

Data was scored on the basis of presence (1) or absence (0) for analysis. If a product was present in a genotype, it was considered as '1' and if absent considered as '0'. The data was maintained in the excel sheet format for further analysis.

The polymorphism percentage was calculated as per the method suggested by Blair *et al.*, (1999).

Polymorphism (%) = (Total number of bands - Number of monomorphic bands)/Total number of bands x 100. The molecular weights of the PCR products were estimated by Alpha Ease FC4.0.0 software (Alpha Innotech Corporation, USA) for each primer to analyse alleles range.

### 3.5.2.8 Data analysis:

The data generated by RAPD were analysed with the software POPGENE 32 version 1.31 (Yeh and Boyle, 1997). Diploid data analysis for dominant marker was performed with the assumption of Hardy-Weinberg equilibrium. Multiple populations were used for the estimation of effective allele number, polymorphic loci, Nei's unbiased measures of genetic identity and genetic distance as well as for dendrogram construction.

**Dendrogram:**

A dendrogram drawn, based on Nei's (1978) genetic distances using UPGMA. This program is an adoption of program NEIGHBOR of PHYLIP version 3.5c by Joe F elsenstein. The drawing was executed for Multiple Populations.

## 3.6 Effect of fungicides, phytoextracts and antagonists on Penicillium fruit rot of citrus *in vitro* and *in vivo*

### 3.6.1 Fungicides

#### 3.6.1.1 *In vitro*

Bio-efficacy of different fungicides were studied *in vitro* by following Poisoned Food Technique method (Nene and Thapliyal, 1979) against Penicillium fruit rot pathogen with different concentrations; Azoxystrobin and azoxystrobin (18.2 %) + difenconazole (11.4 %) at 250 and 500 ppm, mancozeb (75%) at 1000 and 2000 ppm while hexaconazole, hexaconazole (5%) + captan (70%), carbendazim (12%) + mancozeb (63%), cymoxanil (8%) + mancozeb (64%), thiophanate methyl, carbendazim at 500 and 1000 ppm. The list of fungicides screened are given in the Table 3.2 along with their common name, trade name and chemical name. Required quantity of each fungicide under study was mixed thoroughly in sterilized 100 ml PDA media filled in 250 ml flask separately under aseptic condition. The medium was supplemented with

**Table 3.2: Statement showing the common name, trade name and chemical name of fungicides.**

| Sr. No. | Common name | Trade Name | Chemical name |
|---|---|---|---|
| 1 | Hexaconazole(HCZ) | Contaf 5 EC | 2, 4 – triazole-1- ethanol |
| 2 | Hexaconazole (5%) + Captan (70%)(HCZ+CAP) | Taquat 75 WP | 2, 4 – triazole-1- ethanol + N- trichloromethylthio -4-cyclohexene -1, 2- dicaroximide. |
| 3 | Azoxystrobin (ASB) | Amistar 25 SC | Methyl (E)-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate) |
| 4 | Carbendazim (12%) + Mancozeb (63%) (CBZ+MNZ) | Sixer 75 WP | 1 H – Benzimidazol – 2 – ylcarbamic acid methyl ester + (Ethylenebis (dithiocarbamato)) manganese mixture with (ethylenebis (dithiocarbamato)) zinc |
| 5 | Cymoxanil (8%) +Mancozeb (64%)(CXL+MNZ) | Curzate M 8 72 WP | 2- cyno- N- (Ethylamino carbonyl) - 2-(Methoxyiminoacetamide) |
| 6 | Thiophanate methyl(TPM) | Topsin M 70 WP | Dimethyl ester 4,4 – o –phenylenebis (3- thioallophanic) acid |
| 7 | Carbendazim(CBZ) | Bavistin 50 WP | 1 H – Benzimidazol – 2 – ylcarbamic acid methyl ester |
| 8 | Mancozeb(MNZ) | Dithane M-45 75 WP | (Ethylenebis (dithiocarbamato)) manganese mixture with (ethylenebis (dithiocarbamato)) zinc |
| 9 | Azoxystrobin 18.2% + Difenconazole 11.4 %(ASB+DCZ) | Amistar Top 29.6 SC | Methyl (E)-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate) + 1-[2-[4-(4-chlorophenoxy)-2-chlorophenyl]-4-methyl-1, 3-dioxolan-2-ylmethyl]-1H-1, 2, 4-triazole |

streptomycin sulphate @ 50 ppm to prevent bacterial contamination. The poisoned medium was then poured in sterilized Petri plates (20 ml) and allowed it to solidify. The plates were then inoculated with 20 micro litre with spore suspension ($10^6$ spores/ml) of seven days old culture of test pathogens by placing in the centre of the plate. Control was maintained for each set where fungal spore suspension was placed on PDA medium without fungicide. Each treatment was replicated three times. The inoculated plates were then incubated at 25 ± $1^0$ C in BOD incubator for seven days. The observations on mycelial growth (mm) and per cent growth inhibition of test fungi were recorded after 7 days of incubation. The per cent growth inhibition (PGI) of pathogen in each treatment was calculated by following formula (Asalmol *et al.*, 1990).

$$I = \frac{C - T}{C} \times 100$$

Where I = Inhibition per cent

C = Colony diameter (mm) in control plate

T = Colony diameter (mm) in treated plate

### 3.6.1.2 *In vivo*

To study the effect of fungicides *in vivo*, the procedure mentioned earlier in 3.6.1.1 was followed.

#### 3.6.1.2.1 Pre- inoculation

The healthy, matured, uniform size citrus fruits of 'Kagzi lime' cultivar were surface sterilized by dipping in 0.1 per cent NaOCl solution for one min. followed by three successive washings with distilled sterile water and inoculated separately with the pathogen by the wound inoculatin method. The fruits were first inoculated with 25 µl of fruit rot pathogen ($10^6$ spores/ml) suspension using a micropipette. The inoculated and uninoculated (dipped in distilled sterile water) fruits were placed separately in sterilized plastic tray containing sterilized moist absorbent cotton at the bottom and covered with sterilized polythene sheet. The interval between fungicidal treatment and inoculation was kept twelve hours. The severity of fruit rot was recorded on $4^{th}$ and $8^{th}$ day after inoculation with the help of assessment key (Plate 3.1).

#### 3.6.1.2.2 Post- inoculation

The procedure detailed in 3.6.1.2.1 was followed except that the fruits were first inoculated with test pathogen and then treated with fungicides.

### 3.6.2 Phytoextracts

#### 3.6.2.1 *In vitro*

Efficacy of different phytoextracts of plant species having medicinal value were tested *in vitro* by Poisoned

Food Technique against citrus fruit rot pathogen (Nene and Thapliyal, 1979). The list of plant species used for phytoextract study is given in Table 3.3 indicating their botanical name, common name and plant part used. All the phytoextracts were tested at 10 per cent concentration.

Fresh and healthy 100 g plant parts of each species were thoroughly washed with tap water and then with distilled sterile water. They were macerated separately in grinder mixture by adding 100 ml acetone.

The mixture was filtered through two fold sterilized muslin cloth and the filtrate was centrifuged at 5000 rpm for 10 min. and the clear supernatant extract was collected in sterilized conical flasks. After evaporating the Acetone from extract, the clear extract was collected and diluted with 100 ml distilled sterile water to make volume 1:1 (W/V). This was considered as 100 per cent concentration for the study to test the efficacy of plant extracts (Sinha and Saxena, 1989). Each phytoextract (10 %) was mixed thoroughly in sterilized 100 ml PDA medium filled in 250 ml flask under aseptic condition. The medium was supplemented with streptomycin sulphate @ 50 ppm to prevent bacterial contamination. Further procedure was same as followed earlier in 3.6.1.1.

**Table 3.3: List of phytoextracts used for study**

| Sr. No. | Common name | Botanical name | Part used |
|---|---|---|---|
| 1 | Eucalyptus | *Eucalyptus amygdalina* | Leaves |
| 2 | Garlic | *Allium sativum* | Leaves |
| 3 | Henna | *Lawsonia inermis* | Leaves |
| 4 | Turmeric | *Curcuma longa* | Powder |
| 5 | Alovera | *Aloe barbadensis* | Pulp |
| 6 | Tulsi | *Ocimum sanctum* | Leaves |
| 7 | Neem | *Azadirachta indica* | Leaves |
| 8 | Cinnamon | *Cinammomum verum* | Leaves |
| 9 | Lemon grass | *Cymbopogan flexuosus* | Leaves |

### 3.6.2.2 *In vivo*

The procedure detailed in 3.6.2.1 was followed. The phytoextracts studied *in vitro* were tested at 10 per cent concentration by following both pre- and post-inoculation methods.

#### 3.6.2.2.1 Pre- inoculation

In pre-inoculation treatment, the fruits were first dipped in phytoextracts separately and then inoculated with the pathogen by keeping 12 hours interval. Further procedure was followed as mentioned earlier in 3.6.1.2.1.

#### 3.6.2.2.2 Post- inoculation

In the post inoculation method, the fruits were first inoculated with pathogen and then treated with the phytoextracts. The procedure described earlier in 3.6.1.2.1. was followed.

### 3.6.3 Antagonists

#### 3.6.3.1 *In vitro*

Antagonistic effect of different bioagents i.e. *Trichoderma viride, T. harzianum, T. virens, Pseudomonas fluorescens* and *Bacillus subtilis* were tested by dual culture technique for their antagonism against citrus fruit rot pathogen.

Seven days old culture of the bioagents and the pathogen were employed by following dual culture method. Mycelial disc of 5 mm diameter cut from the periphery of both antagonist and test pathogen and were placed at 50 mm apart from each other in Petri plates and in case of bacterial bioagents half portion of plates streaked and 5 mm diameter mycelial disc placed at centre of Petri plates. In control, only test pathogen was kept in the centre of Petri plate. Each treatment was replicated four times. The Petri plates were incubated at 25 ± $1^0$ C in BOD incubator. Observations on per cent growth inhibition were recorded after 7 days of incubation by following the procedure mentioned earlier in 3.6.1.1.

#### 3.6.3.2 *In vivo*

Antagonists studied *in vitro* were further evaluated to test their antagonism in controlling Penicillium fruit rot of citrus following both pre- and post-inoculation methods.

#### 3.6.3.2.1 Pre- inoculation

The healthy semi-ripe fruits were first inoculated with spore suspension ($10^6$ spores/ml) of seven days old culture of different antagonists separately and after 24 hrs, the fruits were inoculated at the same site with spore suspension ($10^6$ spores/ml) of seven days old culture of test pathogen. Control was maintained separately only with pathogen. Each treatment was replicated four times. Further procedure mentioned earlier in 3.6.1.2.1 was followed.

## 3.7. Effect of Hot Water Treatment on Penicillium fruit rot of citrus *in vivo*

In Post inoculation Hot Water Treatment, matured healthy fruits were inoculated with pathogen (25µl of spore suspension) ($10^6$ spores/ ml) and after 3 hours, the inoculated fruits were dipped in a hot water bath at 50 ± 2°C for 3 and 4 minutes and 55 ± 2°C for 2, 3 and 4 minutes then rinsed with distilled sterile water, air-dried and placed in sterilized plastic tray containing sterilized moist absorbent cotton at the bottom and covered with sterilized polythene sheet. The fruits were kept at 25 ± 1°C temperature. The observations on disease severity was recorded after 4th and 8th days of inoculation following standard key.

## 3.8 Effect of Hot Water Treatment on protein patterns (SDS-PAGE) of citrus fruit peel

### 3.8.1 Extraction of protein from citrus fruit

Proteins from treated and untreated citrus fruit peel were extracted by grinding 200 mg of citrus fruit tissue in liquid nitrogen with the help of pestle and mortar and homogenate was obtained by adding 2 ml, lysis buffer. The homogenate was centrifuged for 10 min. at 10,000 rpm at 4°C and supernatant was precipitated by adding 3 volumes of prechilled acetone and stored at -20°C for 2hours. The supernatant centrifuged at 10,000 rpm for 10 min. at $4^0$C to collect the precipitated protein pellet by discarding the aqueous phase.

Air dried Protein pellets were then dissolved in 100 µl of protein sample buffer, this dissolved protein sample was used for further study.

**Materials:**

**A vertical slab electrophoresis unit**

**Stock Acrylamide Solution** **:** (30%) stored at 4°C

| | |
|---|---|
| Acrylamide 30% | : 30 g |
| Bisacrylamide 0.8% | : 0.8 g |
| Distilled water | : 100 ml |

**Separating Gel Buffer** : (pH: 8.8)

| | |
|---|---|
| 1.5 M Tris-HCl | : 18.16 g |
| Distilled water | : 100 ml |

**Stacking Gel Buffer** : (pH: 6.8)

| | |
|---|---|
| 0.5 M Tris-HCl | : 3 g |
| Distilled water | : 50 ml |

**Polymerizing Agents:**

I. Ammonium persulphate 10% (APS) 0.1 g/ml, prepared freshly before use.

II. TEMED (N, N, N', N'–Tetramethylethylene diamine)

**Protein Extraction Buffer**

| | |
|---|---|
| Urea | :7M |
| Thio Urea | :2M |
| Tris HCl | :40mM |

**Sample Buffer:** (pH: 6.8)

| | |
|---|---|
| 0.5 M Tris HCl | : 1.0 ml |
| 10 % SDS | : 1.6 ml |
| 0.5 % Bromophenol blue | : 0.4 ml |
| Glycerol | : 1.6 ml |
| Mercaptoethanol | : 0.4 ml |
| Distilled water | : 10 ml |

**Sample:** 50 µl supernatant was added in 10 µl sample buffer and boiled for 2-4 min at 100 °C in water bath.

**Electrode Buffer:** (pH: 8.2 – 8.4)

| | |
|---|---|
| 0.05 M Tris | : 12 g |
| 0.192 M Glycine | : 28.8 g |
| 0.1% SDS | : 2 g |
| Distilled water | : 2 lit. |

**Protein Stain Solution:**

| | |
|---|---|
| Coomassie brilliant blue R 250 | : 0.1 g |
| Methanol | : 40 ml |
| Acetic acid | : 10 ml |
| Distilled water | : 50 ml |

First, dissolved the dye in methanol and then mixed with acetic acid and distilled water. Fresh preparation was used every time.

**Destainer:** As above without the dye.

**Methodology for SDS-PAGE:**

- Thoroughly cleaned and dried glass plates and spacers, then assembled them properly. Hold the assembly together with bulldog clips. Clamped in an upright position. Two per cent agar was then applied around the edges of the spacers to hold them in place and seal the chamber between the glass plates.
- Prepared 12 % separating gel by mixing the following solutions:

**Separating Gel (12 %):**

| | |
|---|---|
| 30 % Acrylamide mix | : 8.0 ml |
| 1.5 M Tris–HCl pH 8.8 | : 5.0 ml |
| 10 % SDS | : 200 µl |
| 10 % APS | : 200 µl |
| TEMED | : 8 µl |
| Distilled water | : 6.6 ml |

- Gel solution was mixed gently and carefully poured in the chamber spaced between the glass plates. Distilled water layered on top of the gel and left to set for 30 - 45 minutes.
- Stacking gel (5 %) prepared by mixing the following solution

**Stacking Gel (5 %):**

| | |
|---|---|
| 30 % Acrylamide mix | : 0.83 ml |
| 1 M Tris – HCl pH 6.8 | : 0.63 ml |
| 10 % SDS | : 50 µl |
| 10 % APS | : 50 µl |
| TEMED | : 5 µl |
| Distilled water | : 3.4 ml |

- Removed the water from the top of the gel and washed with a little gel solution. Poured the stacking gel mixture, placed the comb carefully in stacking gel and allowed the gel to set for 30 - 45 minutes.
- After the stacking gel has polymerized, removed the comb without distorting the shape of well. Then filled it with electrode buffer and removed the trapped air bubbles at the bottom of the gel. Connected the cathode at the top and turn on the DC – power to check the electrical circuit. The electrode buffer and the plates were kept cooled so that heat generated during the run is dissipated and do not affect the gel and resolution.

- Took up the 50 µl extract from the prepared samples in a micro syringe and carefully injected it into a sample 'well' through the electrode buffer. Marked the position of the well on the glass plates and the presence of bromophenol blue in the sample buffer which facilitated easy loading of the sample. Similarly, loaded a few wells with standard marker proteins in the sample buffer.
- Turned on the current to 10–15 mA for initial 10–15 min. until the sample travel through the stacking gel. The stacking gel helped concentrations of the samples. Then the run was continuing at 30 mA until the bromophenol blue reaches the bottom of the gel, which took about 3 hrs.
- After the run is complete, carefully removed the gel inserted between the plates and immersed in staining solution for at least 3 hrs. or overnight with uniform shaking so that the proteins absorb the coomassie brilliant blue.

Transferred the gel into destaining solution and shaked gently and continuously. Dye that is not bound to proteins thus removed. Changed the destainer frequently, particularly during initial periods, until the background of the gel was colorless. As the proteins of minute quantities are stained frequently, destaining process was stopped at appropriate stage to visualize as many bands as possible.

## 3.9 Statistical analysis

Data obtained from various investigations were subjected to statistical analysis by making use of analysis of variance technique (Steel and Torrie, 1980). The standard methods of analysis of variance for complete randomized design and transformations were used in the experiments. The test of significance among the treatments was worked out by 'F' test. The appropriate standard error (S.Em. ±) was computed in each case. For the treatments effects, which were found to be significant, the critical difference (C.D) at 5 per cent level of probability was worked out to compare two treatment means.

# IV. RESULTS AND DISCUSSION

"Studies on Penicillium fruit rot (*Penicillium citrinum*) of citrus (*Citrus aurantifolia* Swingle) and its management" was carried out in the Department of Plant Pathology, B.A. College of Agriculture, Anand Agricultural University, Sardar Patel vegetable market and Goya Talav market, Anand. The results obtained on various aspects are presented here under.

## 4.1 Collection, isolation, purification, pathogenicity and identification of pathogen causing fruit rot in citrus

### 4.1.1 Collection of samples

Diseased citrus fruits (cv- Kagzi lime) showing typical symptoms of Penicillium rot were collected from Sardar Patel vegetable market, Anand. The infected fruits exihibited soft water soaked lesions. The infected area enlarges and whitish mycelial growth appears on the surface of fruit followed by production of olive green spore mass (Barmore and Brown, 1982) (Plate 4.1).

### 4.1.2 Isolation and purification

The freshly infected citrus fruits brought to the laboratory and were subjected to isolation by tissue isolation technique to obtain the culture of pathogen causing Penicillium fruit rot in citrus. The culture thus obtained was further purified by single spore isolation. The pure culture of the pathogen was maintained by periodical transfer on PDA slants throughout the investigations.

### 4.1.3 Pathogenicity

To prove the Koch's postulates, the isolated pathogen was artificially inoculated on healthy matured citrus fruits following wound inoculation method as per the procedure described earlier in 3.2.2. The fruits inoculated with the test pathogen exhibited typical Penicillum rot symptoms on 4th day of inoculation. The artificially inoculated fruits exhibited water soaked, light brown lesions with soft areas easily punctured by finger pressure. The area enlarges in concentric fashion and white mycelium rapidly become light green to olive green with production of olive green spore mass. The entire surface of the fruit displayed olive green color with powdery mass of spores in eight days (Plate 4.2). With the advancement of infection, fruit rind became more succulent and soft emitting a foul oder. The control fruits without inoculation did not produce any symptoms (Plate 4.2). The Penicillium rot symptoms produced on artificially inoculated fruits were similar to those observed on naturally infected fruits. Further, the reisolations made from artificially inoculated fruits produced whitish mycelial growth covered with olive green spores and was identical to the one which was used for inoculation of the fruits.

Results similar to the present investigation showing typical fruit rot symptoms produced by Penicillium on

artificially inoculated mature orange fruit was reported by Barmore and Brown (1982); Brown and Barmore (1983) and Smilanick *et al.* (2005)

### 4.1.4 Identification

After purification of the fungus, the cultural and morphological characters were studied for identification of the pathogen. The pure culture obtained was sent for identification to Indian Type Culture Collection (I.T.C.C.), Division of Mycology and Plant Pathology, I.A.R.I., New Delhi and was identified as *Penicilliun citrinum* (ID. No. 8298.11). The cultural and morphological characters of the fungus were found similar to that of *Penicillium citrinum* causing Penicillium fruit rot in citrus Oliveri *et al.* (2007). Thus, the causal agent of Penicillium fruit rot of citrus in middle Gujarat was confirmed as *Penicillium citrinum.*

#### 4.1.4.1 Cultural characters

The fungus (*Penicillium citrinum*) produced bluish green mycelial growth with abundant sporulation on PDA covering the petri plate (70 mm) within 7 days of incubation at 25 ± 1° C temperature. In initial stage the fungal growth was white, rapidly becoming light green to bluish olive green with production of conidia. (Plate 4.3)

### 4.1.4.2 Morphological characters

The fungus produces whitish blue septate mycelial growth on PDA, later turned to light green and finally become olive green in color. Branching of mycelium was irregular. The conidophores are branched twice or thrice, elongate, bearing long tangled chaines of conidia. Metulae arising from branches at same level, 3-5 in number and about 10-15 × 3-5µ phialides in groups of 5-9. Conidia are smooth and spherical with 2-3 µ in diameter [Plate 4.4 (a & b)].

## 4.2 Survey of post harvest diseases of citrus at Sardar Patel vegetable market, station road and Goya Talav market at Anand.

The weekly survey was carried out from first week of October, 2010 to fourth week of March, 2011 revealed the presence of bacterial canker, Penicillium, Aspergillus, Phytophthora, Alternaria, Stem end rot and others (Plate 4.5) at Sardar Patel vegetable market, station road and Goya Talav market, Anand.

The data presented in Table 4.1 and Fig. 4.1 indicated that at Sardar Patel vegetable market, incidence of Penicillium rot was predominant (6.48 %) followed by bacterial canker (4.79 %) and Aspergillus rot (3.88 %). The incidence of Phytophthora rot, other fruit rots, Stem end rot and Alternaria rots were 0.65, 0.42, 0.12, 0.10 per cent, respectively.

## Table 4.1: Incidence of citrus fruit rots at Sardar Patel vegetable market, station road, Anand

| Sr. No. | Fruit rots | Percent Disease Incidence | | | | | | | | | | | | | | | | | | | | | | | | Mean |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | October | | | | November | | | | December | | | | January | | | | February | | | | March | | | | |
| | | week | | | | week | | | | week | | | | week | | | | week | | | | week | | | | |
| | | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | |
| 1 | Bacterial canker | 14.5 | 13.3 | 11.6 | 11.1 | 11.5 | 11.1 | 10.9 | 10.2 | 9.8 | 9.3 | 1.2 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 4.79 |
| 2 | Penicillium rot | 0.5 | 0.5 | 0.9 | 1.4 | 2.95 | 3.75 | 3.75 | 5.6 | 6.0 | 6.3 | 7.0 | 7.7 | 8.2 | 8.9 | 9.1 | 9.5 | 11.0 | 10.5 | 10.3 | 9.9 | 9.1 | 6.2 | 4.1 | 1.25 | 6.48 |
| 3 | Aspergllus rot | 8.3 | 7.9 | 7.4 | 7.0 | 6.9 | 6.6 | 6.4 | 6.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 8.3 | 8.8 | 9.5 | 9.9 | 3.88 |
| 4 | Alternaria rot | 0.4 | 0.5 | 0.8 | 0.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.10 |
| 5 | Stem-end rot | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.5 | 0.52 | 0.8 | 0.95 | 0.12 |
| 6 | Phytophthora rot | 3.2 | 2.9 | 2.8 | 2.2 | 2.0 | 1.8 | 0.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.65 |
| 7 | Others | 0.8 | 0.8 | 0.9 | 0.99 | 0.5 | 0.6 | 0.69 | 0.7 | 0.5 | 0.48 | 0.42 | 0.39 | 0.38 | 0.35 | 0.32 | 0.29 | 0.0 | 0.0 | 0.0 | 0.0 | 0.28 | 0.26 | 0.23 | 0.2 | 0.42 |
| | Total | 27.7 | 25.9 | 24.4 | 23.3 | 23.9 | 23.9 | 22.3 | 22.6 | 16.3 | 16.1 | 8.62 | 8.59 | 8.58 | 9.25 | 9.42 | 9.79 | 11.0 | 10.5 | 10.3 | 9.9 | 18.2 | 15.8 | 14.6 | 12.3 | |

The highest incidence of bacterial canker (14.5 %) was recorded in 1st week of October, while peak incidence of Penicillium rot (11.0 %) was recorded in 1st week of February, Aspergillus rot (9.90 %) in 4th week of March, Phytophthora rot (3.20 %) in 1st week of October, Stem end rot (0.95 %) in 4th week of March and Alternaria rot (0.8 %) in 3rd week of October. Overall, highest rot incidence (27.7 %) due to various pathogens was recorded in 1st week of October.

The data presented in Table 4.2 and Fig. 4.2 revealed that during 2010-11 at Goya Talav market, incidence of Penicillium rot was predominant (5.95 %) followed by bacterial canker (4.74 %) and Aspergillus rot (3.78 %). The incidence of Phytophthora rot, other fruit rots, Stem end rot and Alternaria rot were 0.63, 0.41, 0.11, 0.08 per cent, respectively.

The highest incidence of bacterial canker (13.7%) was recorded in 1st week of October, while peak incidence of Penicillium rot (10.9%) was recorded in 2nd week of February, Aspergillus rot (9.7%) and Stem end rot (0.9 %) in 4th week of March, Alternaria rot (0.6 %) and Phytophthora rot (3.0 %) in 1st week of October. Overall, highest rot incidence (26.7 %) due to various pathogens was recorded in 1st week of October. Disease incidence of various rots was found maximum at Sardar Patel vegetable market, station road, rather than Goya Talav vegetable market, Anand. Thus, at both the locations Penicillium rot was found predominant over all other rots during the period of survey.

## Table 4.2: Incidence of citrus fruit rots at Goya talav market, station road, Anand

| Sr. No. | Fruit rots | Percent Disease Incidence | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | October | | | | November | | | | December | | | | January | | | | February | | | | March | | | | Mean |
| | | week | | | | week | | | | week | | | | week | | | | week | | | | week | | | | |
| | | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | 1 | 2 | 3 | 4 | |
| 1 | Bacterial canker | 13.7 | 13.1 | 11.6 | 11.5 | 11.1 | 11 | 10.9 | 10.2 | 9.8 | 9.3 | 1.2 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 4.74 |
| 2 | Penicillium rot | 0.5 | 0.5 | 0.9 | 1.4 | 2.95 | 3.75 | 3.75 | 5.6 | 6.0 | 6.3 | 7.0 | 7.4 | 8.2 | 8.9 | 9.1 | 9.5 | 10.1 | 10.9 | 10 | 9.9 | 8.6 | 6.2 | 4.1 | 1.25 | 5.95 |
| 3 | Aspergillus rot | 8.1 | 7.9 | 7.2 | 7.0 | 6.9 | 6.6 | 5.6 | 5.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 8.3 | 8.8 | 9.5 | 9.7 | 3.78 |
| 4 | Alternaria rot | 0.6 | 0.5 | 0.5 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.08 |
| 5 | Stem-end rot | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.5 | 0.52 | 0.75 | 0.9 | 0.11 |
| 6 | Phytophthora rot | 3.0 | 2.8 | 2.8 | 2.1 | 2.0 | 1.8 | 0.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.63 |
| 7 | Others | 0.8 | 0.75 | 0.9 | 0.9 | 0.5 | 0.6 | 0.69 | 0.7 | 0.5 | 0.48 | 0.42 | 0.39 | 0.38 | 0.35 | 0.32 | 0.29 | 0.0 | 0.0 | 0.0 | 0.0 | 0.28 | 0.24 | 0.23 | 0.2 | 0.41 |
| | Total | 26.7 | 25.6 | 23.9 | 23.3 | 23.5 | 23.8 | 21.5 | 21.7 | 16.3 | 16.1 | 8.62 | 8.29 | 8.58 | 9.25 | 9.42 | 9.79 | 10.1 | 10.9 | 10 | 9.9 | 17.7 | 15.8 | 14.6 | 12.1 | |

The fruits are injured during harvesting, grading, packaging and transportation, which make major avenues for infection by various pathogens. Further, more number of cooler days and high humidity with morning dew provide more conducive conditions for infection by the pathogens and this leads to deterioration of the fruits.

Naqvi and Daas (1994) surveyed the several districts of Maharastra and reported 43 and 47 per cent losses in mandarins due to post harvest diseases (*Botrydiplodia theobromea, Colletotrichum gleosporioides*, *Alternaia citri, Phomopsis citri*, *Geotrichum candidum*, *Penicillium digitatum* and *P. italicum*) when transported through truck and train, respectively.

Kaur and Verma (2002) surveyed the prevalence of post harvest rots of kinnow in Punjab, where the incidence of *Penicillium digitatum* (58.33%) was identified as the major post harvest disease followed by *Alternaria citri* (33.3%), *Penicillium italicum* (26.7%), *Aspergillus niger* (20%) and *Phytophthora* sp. (13.3%).

Singh and Thakur (2003) observed the *Penicillium digitatum* as the most predominant post harvest fungal pathogen followed by *Alternaria citri* and *P. italicum* during the storage of kinnow fruits.

Singh and jain (2004) studied the losses of Kinnow mandarins due to mycoflora in long distance marketing from Punjab to Banglore, where they recorded highest incidence of *Alternaia alternata* (81.1%) followed by *Penicillium digitatum* (64%) and *Penicillium italicum* (70%).

Verma (2009) observed major fruit spoilage of Kinnow mandarins from November to April and which was dominated by incidence of *Penicillium digitatum* (5.18-8.85%) and *P. italicum* (2.59-4.82%) with peak losses in January (8.25%) – February (8.85%) and February (4.82%) – March (4.43%), respectively.

## 4.3 Studies on cell wall degrading enzymes.

The activities of PG and PMG were studied in matured fruits inoculated with *Penicillium citrinum* and healthy fruits. The enzymatic activity of PG was higher in fruits inoculated with *P. citrinum* (33.32 %) than in healthy fruits (9.29 %). The enzymatic activity of PG was found significantly increased with time. The maximum reduction in viscosity was observed in PG at 120 min (68.35 %) (Table 4.3 and Fig. 4.3). The interaction effect between stage of fruit ripeness and period found significant.

Significant differences were not observed in the activity of PMG between inoculated (*P. citrinum*) and healthy fruit peel. The enzymatic activity of PMG in Penicillium inoculated fruits was found at par with healthy fruits at all intervals.

**Table 4.3: Impact of time duration on synthesis of cell wall degrading enzymes (PG & PMG) by *P. citrinum***

| Stage | Per cent reduction in viscosity | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Polygalacturonase (at min) | | | | Polymethylgalacturonase (at min) | | | |
| | 10 | 30 | 120 | Mean | 10 | 30 | 120 | Mean |
| **Inoculated** | 9.25 | 22.36 | 68.35 | 33.32 | 2.64 | 6.28 | 19.35 | 9.42 |
| **Healthy** | 2.57 | 6.22 | 19.07 | 9.29 | 2.65 | 6.25 | 19.09 | 9.33 |
| **Mean** | 5.91 | 14.29 | 43.71 | | 2.65 | 6.27 | 19.22 | |
| **Source** | **SEm±** | **CD** | | | **SEm ±** | **CD** | | |
| **Stage (S)** | 0.14 | 0.44 | | | 0.08 | NS | | |
| **Period (P)** | 0.17 | 0.54 | | | 0.18 | NS | | |
| **S X P** | * | * | | | NS | NS | | |
| **CV %** | **2.05** | | | | **2.82** | | | |

Results similar to the present investigation was reported by Patil and Pathak (1994). They found that activity of polygalacturonase (PG) and cellulolytic enzymes (CX) were highest at all intervals (10, 30 and 120 min.) in ripe mango fruits compared to semi ripe fruits when inoculated with *Botryodiplodia theobromae* and *Rhizopus arrhizus*.

Barmore and Brown (1979) recorded 51.7 per cent reduction in viscosity of exo-poly galacturonase enzyme in orange fruits infected with *Penicillium digitatum* at 45 min. of incubation.

The incubation period required for 50 per cent reduction in viscosity employing 0.5 enzyme units was 230 min for PG –I and PG –II (Hershenhorn, *et al.*1990).

Bandyopadhyay and Chaudhuri (2004) observed 97.3 per cent reduction in viscosity of PG enzyme in the banana fruit infected by *Penicillium* sp.

## 4.4 Molecular variability among the isolates of *Penicillium citrinum* causing mold rot in citrus fruit using RAPD markers

### 4.4.1 Assay of DNA from the isolates of *Penicillium* spp. obtained through Nanodrop Technique.

The results of Spectrophotometric DNA analysis showed 1100, 1225, 405, 985 and 922 ng/µl DNA concentration in mycelia of AND, VDA, ABD, DKR and NAV isolates, respectively. The absorbance ratio of DNA at 260/280 wave length was 1.87, 1.85, 1.86, 1.88 and 1.85 for AND, VDA, ABD, DKR and NAV isolates, respectively (Table 4.4). Extracted DNA was used for molecular characterization through RAPD primers.

**Table 4.4: Assay of DNA from the *Penicillium* isolates obtained through "Nanodrop Technique".**

| Isolates | ( 260/280) Wavelength Ratio | Conc. (ng/µl) |
|---|---|---|
| AND | 1.87 | 1100 |
| VAD | 1.85 | 1225 |
| ABD | 1.86 | 405 |
| DKR | 1.88 | 985 |
| NAV | 1.85 | 922 |

### 4.4.2 Random Amplification of Polymorphic DNA (RAPD) Study:

The present study showed a high level of genetic variability between the isolates of *Penicillium.* The highest

polymorphism (100%) was showed by the primer OPA-10, OPA-13, OPE-17 and OPE-18. Whereas, the lowest polymorphism (60 %) was observed in the primer OPA-18.

Analysis of the DNA of five isolates with 15 primers showed amplification of total 160 bands, with a range of 16 to 7 bands per primer. Overall, *Penicillium* isolates exhibited a moderate level of genetic diversity. The maximum 16 bands were generated by a single primer (primer 0PA- 12); whereas, primer OPA-11, OPE-14, OPE-15, OPE-3 and OPA-9 generated 15, 14, 14, 12 and 11 bands respectively. Similarly, primer 0PA-13, OPA-18 and OPE-17 generated 10 bands each, primer 0PE-18 and OPE-11 generated 9 bands. However, primer 0PA-5 and OPE-12 generated only 8 bands. The lowest number of bands were generated by primer OPA-1 and OPA-10 (7 bands). This result showed the ability of RAPD to discriminate among isolates and suggested their application for species identification.

A total of 40 decamer primer belonging to OPE and OPA series were screened for RAPD analysis. Of these, 15 primers were selected based on repeatability. RAPD markers proved to be very informative and useful in monitoring the genetic diversity in the biological entity. Therefore, this technique was employed to detect variability present among five isolates i.e. AND, VDA, ABD, DKR and NAV of *Penicillium*

*citrinum*. The fragment sizes were detected by comparing the amplicons with a 1 kbp DNA Ladder. The purpose of this study was to identify the specific primers which are likely to be efficient in revealing the diversity among the isolates of *P. citrinum.* The results obtained using 15 primers have been presented in Table 4.5

A common dendrogram which was generated using the Pop Gene 32 software has been collectively discussed for all the15 primers.

**1. Primer OPA-1 (AAT CGG GCT G):**

A maximum of 7 PCR DNA fragments (Plate 4.6) were observed in OPA-1 primer. This primer showed 6 polymorphic bands out of the 7 bands thereby, giving 85.71 % polymorphism (Table 4.5). The size ranged from 412 bp to 5341 bp.

**2. Primer OPA-5 (GTG ACG TAG G):**

A maximum of 8 PCR DNA fragments (Plate 4.6) were observed with primer OPA-5. This primer showed 5 polymorphic band out of the 8 bands thereby, giving 62.50 % polymorphism (Table 4.5). The size ranged from 307 bp to 5824 bp.

**3. Primer OPA-9 (GGG TAA CGC C):**

The primer OPA-9 generated 11 DNA fragments (Plate 4.6). This primer showed 10 polymorphic bands out of the 11 bands, thereby, giving 90.90 % polymorphism (Table 4.5). The size ranged from 409 bp to 7208 bp.

**4. Primer OPA-10 (GTG ATC GCA G):**

The primer OPA-10 generated 7 DNA fragments (Plate 4.6) and the size ranged from 630 bp to 5917 bp. This primer showed 7 polymorphic bands out of the 7 bands, thereby, giving 100% polymorphism (Table 4.5).

**5. Primer OPA-11 (CAG CAC CCA C):**

The primer OPA-11 generated maximum 15 DNA fragments (Plate 4.6) and the size ranged from 422 bp to 6523 bp. This primer showed 12 polymorphic bands out of the 15 bands, thereby, giving 80.00 % polymorphism (Table 4.5).

**6. Primer OPA-12 (AGG TGA CCG T):**

The primer OPA-12 generated 16 DNA fragments (Plate 4.6) and the size ranged from 459 bp to 7243 bp. This primer showed 13 polymorphic bands out of the 16 bands, thereby, giving 81.25 % polymorphism (Table 4.5).

**7. Primer OPA-13 (GTT GCG ATC C):**

The primer OPA-13 generated 10 DNA fragments (Plate 4.6) and the size ranged from 353 bp to 5237 bp. This primer showed 10 polymorphic bands out of the 10 bands, thereby, giving 100 % polymorphism (Table 4.5).

**8. Primer OPA- 18 (CCA GAT GCA C):**

The primer OPA-18 had generated a maximum of 10 DNA fragments (Plate 4.6) and the size ranged from 487 bp to

6847 bp. Out of 10 bands obtained, 6 bands were polymorphic, thereby, giving 60.00 % polymorphism (Table 4.5).

**9. Primer OPE- 3 (GTG ACA TGC C):**

A maximum of 12 DNA fragments (Plate 4.7) were observed when primer OPE-3 was used. Out of 12 bands obtained, 9 bands were polymorphic, thereby, giving 75.00 % polymorphism (Table 4.5). The size ranged from 430 bp to 4767 bp.

**10. Primer OPE-11 (AGA TGC AGC A) :**

A maximum of 9 DNA fragments (Plate 4.7) were observed when primer OPE-11 was used. Out of 9 bands obtained, 8 bands were polymorphic, thereby, giving 88.88 % polymorphism (Table 4.5). The size ranged from 397 bp to 5830 bp.

**11. Primer OPE-12 (CCC GAT TCG G):**

A maximum of 8 DNA fragments were observed (Plate 4.7) and the size ranged from 876 bp to 4790 bp when primer OPE-12 was used. Out of 8 bands obtained, 6 bands were polymorphic thereby, giving 75.00 % polymorphism (Table 4.5).

**12. Primer OPE-14 (TGC GGC TGA G):**

The primer OPE-14 generated 14 DNA fragments (Plate 4.7) and the size ranged from 489 bp to 6654 bp. This

primer showed 11 polymorphic bands out of the 14 bands, thereby, giving 78.57 % polymorphism (Table 4.5).

**13. Primer OPE-15 (ACG CAC AAC C):**

The primer OPE-15 generated 14 DNA fragments (Plate 4.7) and the size ranged from 710 bp to 8120 bp. This primer showed 12 polymorphic bands out of the 14 bands, thereby, giving 85.71 % polymorphism (Table 4.5).

**14. Primer OPE-17 (ACG GCG TAT G) :**

Total 10 PCR DNA fragments (Plate 4.7) were observed when primer OPE-17 was used. This primer gave 100 % polymorphism by giving 10 out of 10 polymorphic bands (Table 4.5). The size ranged from 754 bp to 5307 bp.

**15. Primer OPE-18 (AAC GGT GAC C):**

A maximum of 9 PCR DNA fragments were observed (Plate 4.7) and the size ranged from 219 bp to 6407 bp when primer OPE-18 was used. This primer gave 100 polymorphism with 9 polymorphic bands (Table 4.5).

### 4.4.3 Pooled RAPD

Dendrogram (Figure 4.4) based on "Nei's (1978) unbiased measures of genetic distance by UPGMA method" formed two clusters. Cluster 1 includes four isolates *viz.*, isolate AND, ABD, DKR, and NAV cluster 2 includes single isolate VDA.

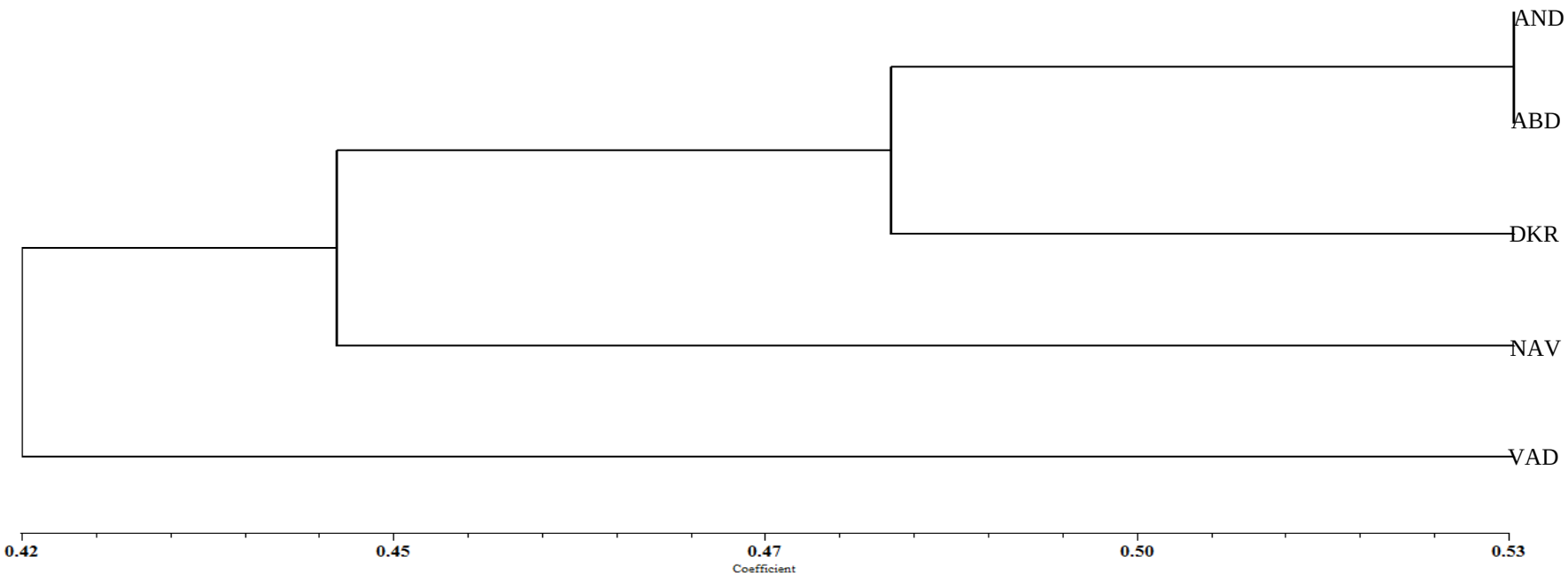


**Fig. 4.4: Dendrogram of five isolates of *Penicillium* based on Nei's (1978) similarity coefficient using UPGMA as the clustering method for RAPD**

## Table 4.5: Per cent polymorphism in RAPD

| Primer | Primer sequence | No. of bands | No. of Polymorphic bands | Polymorphism (%) |
|---|---|---|---|---|
| OPA-1 | AAT CGG GCT G | 7 | 6 | 85.71 |
| OPA-5 | GTG ACG TAG G | 8 | 5 | 62.50 |
| OPA-9 | GGG TAA CGC C | 11 | 10 | 90.90 |
| OPA-10 | GTG ATC GCA G | 7 | 7 | 100 |
| OPA-11 | CAG CAC CCA C | 15 | 12 | 80.00 |
| OPA-12 | AGG TGA CCG T | 16 | 13 | 81.25 |
| OPA-13 | GTT GCG ATC C | 10 | 10 | 100 |
| OPA- 18 | CCA GAT GCA C | 10 | 6 | 60.00 |
| OPE- 3 | GTG ACA TGC C | 12 | 9 | 75.00 |
| OPE-11 | AGA TGC AGC A | 9 | 8 | 88.88 |
| OPE-12 | CCC GAT TCG G | 8 | 6 | 75.00 |
| OPE-14 | TGC GGC TGA G | 14 | 11 | 78.57 |
| OPE-15 | ACG CAC AAC C | 14 | 12 | 85.71 |
| OPE-17 | ACG GCG TAT G | 10 | 10 | 100 |
| OPE-18 | AAC GGT GAC C | 9 | 9 | 100 |
| Total | | **160** | **134** | **83.75** |

The similarity coefficient ranged from 0.42 to 0.53 with all the 15 primers. The maximum genetic similarity was found between the isolate AND and ABD included under one cluster. Whereas, The least Genetic similarity was found between the isolate VDA and AND included in two different clusters. Isolate AND and ABD are more genetically similar than isolate AND and DKR, isolate AND and NAV, the isolate VDA was found genetically more dissimilar than all four (AND, ABD, DKR and NAV) isolates.

In the present study a total of 40 primers comprising of 20 OPA and 20 OPE series were screened against isolates of *Penicillium.* Out of these 15 primers were found useful for amplification of DNA of *Penicillium.* Among the 15 primers the primer viz., OPA-13, OPA-10, OPE-17 and OPE-18 showed 100 per cent polymorphism, followed by OPA-9 (90.90 %), OPE-11 (88.88 %), OPA-1 and OPE-15 (85.71 %) polymorphism of the DNA which helped to ascertain variability. Therefore, these primers could be very useful for ascertaining variability among the population of other species of *Penicillium.*

Araujo *et al.* (2007) were performed RAPD analysis for *P. expansum* and *P. griseoroseum* using 78 single primers of arbitrary sequences. These species presented the differences in banding patterns using only 8 primers. The 54 per cent of

amplified fragments were polymorphic. In spite of small number of amplified fragments between *P. expansum* and *P. griseoroseum*, the results revealed differences in genome of these two species.

Studies on RAPD marker against *A. solani* was done by Varma *et al.* (2007) and Kumar *et al.* (2008) by using primers OPE-2, OPE-3, OPE-4, OPE-5, OPE-6, OPE-7, OPE-8, OPE-10, OPE-11, OPE-12, OPE-14 and OPE-15.

It is concluded that population of *Penicillium* spp. can be screened by this method to ascertain the races. The virulent races of *Penicillium* spp. can be ascertained by using the OPA 13, OPA-10, OPE 18 and OPE 17 primers which had shown 100 per cent polymorphism.

## 4.5 Bio-efficacy of fungicides, phytoextracts and antagonists against Penicillium fruit rot of citrus *in vitro* and *in vivo*

### 4.5.1 Fungicides

#### 4.5.1.1 *In vitro*

Nine fungicides with different concentrations along with control were screened to study their effect on mycelial growth of *P. citrinum in vitro* following standard poison food technique method (Nene and Thapliyal, 1979). The observations on mycelial growth and per cent growth inhibition (PGI) recorded after seven days of incubation and the results obtained are presented in Table 4.6, Fig. 4.5, and Plate 4.8, 4.9.

All the fungicides, screened were found significantly superior in inhibiting the mycelial growth of *P. citrinum* over control.

Out of nine fungicides, seven fungicides screened Azoxystrobin and azoxystrobin 18.2% + difenconazole. (250 and 500 ppm), mancozeb (1000 and 2000 ppm), hexaconazole (5%) + captan (70%), carbendazim (12%) + mancozeb (63%), cymoxanil (8%) + mancozeb (64%), carbendazim (500 and 1000ppm) were found significantly superior in inhibiting mycelial growth of *P. citrinum* (100 %) over control. While hexaconazole and thiophanate methyl (500 and 1000 ppm) gave mediocre effect in inhibiting the mycelial growth (20.45 %, 22.35 % and 17.86 %, 19.92 %) at 500 and 1000ppm concentration, respectively.

Results similar to the present findings have been reported by Godara (1994). Bavistin at 1000 ppm conc. completely inhibited the spore germination of *Penicillium italicum* and *Botryodiplodia theobromae* infecting sweet oranges.

Raju and Naik (2006) observed that carbendazim (12%) + mancozeb (63%) at 500 ppm, copper oxy chloride, cymoxanil (8%) + mancozeb (64%) and mancozeb at 1000 ppm gave 100, 100, 78.96 and 74.12 per cent mycelial growth inhibition of *P. digitatum* in onion, respectively. Javed *et al.* (1995) reported that thiobendazole (0.4%) found most effective in

**Table 4.6: Bio-efficacy of fungicides on mycelial growth and percent growth inhibition of *P. citrinum in vitro***

| Sr. No. | Treatment | Conc. (ppm) | Mycelial growth (mm) | Percent Growth Inhibition (PGI) |
|---|---|---|---|---|
| 1 | Azoxystrobin (ASB) | 250 | 00.00 | 100.00 |
| | | 500 | 00.00 | 100.00 |
| 2 | Azoxystrobin 18.2% + Difenconazole 11.4 % (ASB+DCZ) | 250 | 00.00 | 100.00 |
| | | 500 | 00.00 | 100.00 |
| 3 | Carbendazim (CBZ) | 500 | 00.00 | 100.00 |
| | | 1000 | 00.00 | 100.00 |
| 4 | Carbendazim (12%) + Mancozeb (63%) (CBZ+MNZ) | 500 | 00.00 | 100.00 |
| | | 1000 | 00.00 | 100.00 |
| 5 | Cymoxanil (8%) + Mancozeb (64%) (CXL+MNZ) | 500 | 00.00 | 100.00 |
| | | 1000 | 00.00 | 100.00 |
| 6 | Hexaconazole (5%) + Captan (70%) (HCZ+CAP) | 500 | 00.00 | 100.00 |
| | | 1000 | 00.00 | 100.00 |
| 7 | Hexaconazole (HCZ) | 500 | 52.50 | 20.45 |
| | | 1000 | 51.25 | 22.35 |
| 8 | Thiophanate methyl (TPM) | 500 | 54.21 | 17.86 |
| | | 1000 | 52.85 | 19.92 |
| 9 | Mancozeb (MNZ) | 1000 | 00.00 | 100.00 |
| | | 2000 | 00.00 | 100.00 |
| 10 | Control | - | 66.00 | 00.00 |
| | S.Em. ± | 0.653 | | |
| | C.D. at 5% | 1.87 | | |
| | C.V. (%) | 7.73 | | |

inhibiting the mycelial growth of *A. niger, A. citri, P. digitatum and P. italicum* infecting citrus fruits. Prochloraz (5 ppm) carbendazim (50 ppm) and carbendazim12% + mancozeb 63% (250 ppm) gave complete mycelial growth inhibition of *P. italicum* infecting kinnow mandarin ( Maharshi *et al.*, 2009).

#### 4.5.1.2 *In vivo*

All the fungicides were found significantly superior in reducing the Penicillium rot severity over control on 4th and 8th days after inoculation in pre- and post-inoculation treatments at both the concentrations. (Table 4.7).

#### 4.5.1.2.1 Pre-inoculation method

The results presented in Table 4.7 and Fig. 4.6 revealed that after 4th day of inoculation, significantly lowest rot severity was recorded in fruits treated with mancozeb (2000 ppm) (0.20 %), carbendazim, (1.10 & 0.29 %) followed by carbendazim (12 %)+ mancozeb (63 %) (1.37 & 0.39 %) and cymoxanil (8 %) + mancozeb (64 %) (2.43 & 0.62 %) at 500 and 1000 ppm concentrations, respectively. Thiophante methyl (10.50 & 3.09 %) and hexaconazole (11.70 & 3.56 %) found least effective in controlling the rot at both the concentrations.

Similar trend of results were recorded on 8th day after inoculation. Mancozeb (2000 ppm) (1.40 %) and carbendazim

(3.22 & 1.49 %) at 500 and 1000 ppm proved significantly superior over all other treatments followed by carbendazim (12 %)+ mancozeb (63 %) (4.37 & 2.13 %) and cymoxanil (8 %) + mancozeb (64 %) (6.82 & 3.20 %) at 500 and 1000 ppm, respectively. Thiophante methyl (34.00 & 21.00 %) and hexaconazole (39.20 & 23.80 %) proved least effective in controlling the rot.

#### 4.5.1.2.2 Post-inoculation method

The results presented in Table 4.7 and Fig 4.6 revealed that after 4th day of inoculation, significantly lowest rot severity was recorded in fruits treated with mancozeb (2000 ppm) (1.12 %), carbendazim, (3.35 & 1.34 %) followed by carbendazim (12 %) + mancozeb (63 %) (4.37 & 1.62 %) and cymoxanil (8 %) + mancozeb (64%) (5.40 & 2.70 %) at 500 and 1000 ppm, respectively. Thiophante methyl (25.33 & 14.00 %) and hexaconazole (28.30 & 18.30 %) found least effective in controlling the rot at both the concentrations.

Similar trend of results were recorded on 8th day after inoculation. Mancozeb (2000ppm) (2.70 %), carbendazim, (4.19 & 2.90 %) at 500 and 1000 ppm proved significantly superior over all other treatments followed by carbendazim (12 %) + mancozeb (63 %) (5.70 & 3.98 %) and cymoxanil (8%) + mancozeb (64%) (8.20 & 5.90 %) at 500 and 1000 ppm

**Table 4.7: Effect of fungicides on the severity of Penicillium fruit rot of citrus *in vivo***

| Sr. No | Fungicide | Penicillium rot severity (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Pre-inoculation | | | | Post-inoculation | | | |
| | | 250 ppm | | 500 ppm | | 250 ppm | | 500 ppm | |
| | | 4th | 8th | 4th | 8th | 4th | 8th | 4th | 8th |
| 1 | Azoxystrobin 18.2% + Difenconazole 11.4 % (ASB+DCZ) | 5.10 | 16.20 | 1.47 | 9.62 | 16.80 | 19.40 | 7.40 | 14.12 |
| 2 | Azoxystrobin (ASB) | 6.20 | 21.06 | 1.91 | 13.60 | 20.10 | 25.30 | 10.50 | 16.70 |
| | | 500 ppm | | 1000 ppm | | 500 ppm | | 1000 ppm | |
| 3 | Carbendazim (CBZ) | 1.10 | 3.22 | 0.29 | 1.49 | 3.35 | 4.19 | 1.34 | 2.90 |
| 4 | Carbendazim (12%) + Mancozeb (63%) (CBZ+MNZ) | 1.37 | 4.37 | 0.39 | 2.13 | 4.37 | 5.70 | 1.62 | 3.98 |
| 5 | Cymoxanil (8%) + Mancozeb (64%) (CXL+MNZ) | 2.43 | 6.82 | 0.62 | 3.20 | 5.40 | 8.20 | 2.70 | 5.90 |
| 6 | Hexaconazole (5%) + Captan (70%) (HCZ+CAP) | 3.03 | 8.42 | 0.76 | 6.50 | 10.18 | 10.10 | 4.20 | 7.83 |
| 7 | Thiophanate methyl (TPM) | 10.50 | 34.00 | 3.09 | 21.00 | 25.33 | 40.80 | 14.00 | 29.40 |
| 8 | Hexaconazole (HCZ) | 11.70 | 39.20 | 3.56 | 23.80 | 28.33 | 47.00 | 18.30 | 31.60 |
| | | 1000 ppm | | 2000 ppm | | 1000 ppm | | 2000 ppm | |
| 9 | Mancozeb (MNZ) | 3.20 | 9.08 | 0.20 | 1.40 | 7.00 | 11.38 | 1.12 | 2.70 |
| 10 | Control | 24.30 | 96.30 | 28.75 | 95.00 | 32.00 | 95.00 | 27.00 | 98.00 |
| **S.Em. ±** | | **0.87** | **1.16** | **0.10** | **0.61** | **0.53** | **1.39** | **0.57** | **1.08** |
| **C.D. at 5 %** | | **2.58** | **3.43** | **0.31** | **1.8** | **1.56** | **4.1** | **1.7** | **3.19** |
| **C.V. %** | | **12.75** | **8.44** | **8.44** | **6.59** | **6.1** | **9.01** | **10.3** | **8.92** |

concentrations, respectively. thiophante methyl (40.80 & 29.40 %) and hexaconazole (47.00 & 31.66 %) proved least effective in controlling the rot.

Results similar to the present study are reported by Maharshi *et al.* (2009). Prochloraz (500 ppm) and carbendazim (1000 ppm) gave complete control of blue mold rot (*Penicillium italicum*) of kinnow mandarin followed by carbendazim12 % + mancozeb 63 % (1000 ppm) in both pre and post inoculation treatments.

Verma and Tikoo (2003) reported pre inoculation treatment of thiobendazole, benomyl and carbendazim each at 0.05 per cent found most effective against *Penicillium digitatum* infecting mandarin orange than post inoculation treatment.

Pre harvest sprays of saaf (0.1 %), benomyl (0.1 %), carbendazim (0.1 %) and mancozeb (0.2 %) gave 100, 100, 87.4 and 73.38 per cent disease reduction over control, respectively at 15 days of storage against *Penicillium digitatum* on onion under storage conditions (Raju and Naik 2006).

Carbendazim at 1000 ppm gave 100 per cent control of *Penicillium italicum* in kinnow fruirts upto 60 days in storage followed by mancozeb (1000 ppm) (89 %) over control (Singh and Thakur, 2005).

Rathod and Patel (2005) reported that carbendazim at 500 and 1000 µg/ml and mancozeb at 2000 and 4000 µg/ml found most effective against the Colletotrichum, Penicillium and Alternaria rots in aonla fruits both in Pre and Post inoculation treatments.

Yadav (2009) observed that the application of bavistin (0.05 %) and kavach (0.2 %) found significantly effective in managing the Penicillium rot in aonla fruits.

### 4.5.2 Phytoextracts

#### 4.5.2.1 *In vitro*

Nine phytoextracts (10 %) were tested against the mycelial growth of *P. citrinum in vitro* following standard poison food technique (Nene and Thapliyal, 1979). The observations on the mycelial growth were recorded after seven days of incubation and the results achieved are presented in Table 4.8, Fig. 4.7 and Plate 4.10.

All the phytoextracts screened were found significantly superior in inhibiting the mycelial growth of *P. citrinum* over control. Tulsi, garlic, henna extracts completely inhibited the mycelial growth (100 %) over control. The next best treatment in order of merit were cinnamon (9.00 mm), neem (12.30 mm) and lemon grass (20.00 mm) showing 86.15, 81.80 and 69.23 per cent growth inhibition, respectively over control

(65.00 mm) (Fig. 4.11). Turmeric extract (34.00 mm) found least effective in inhibiting the mycelial growth of the *P. citrinum* (47.69 %).

The results of present investigations corroborate with the results reported by Nath *et al.* (2012). Fifty per cent extract of *Ocimum sanctum* gave 96.50 per cent inhibition of spore germination of *Penicillium brevipactum* infecting kinnow mandarin.

**Table 4.8: Bio-efficacy of Phytoextracts on mycelial growth and percent growth inhibition of *P. citrinum in vitro***

| Sr. No. | Phytoextracts | Mycelial growth (mm) | Percent Growth Inhibition (PGI) |
|---|---|---|---|
| 1 | Tulsi | 00.00 | 100.0 |
| 2 | Garlic | 00.00 | 100.0 |
| 3 | Henna | 00.00 | 100.0 |
| 4 | Cinnamon | 9.00 | 86.15 |
| 5 | Neem | 12.30 | 81.80 |
| 6 | Lemongrass | 20.00 | 69.23 |
| 7 | Aloevera | 27.00 | 58.46 |
| 8 | Eucalyptus | 30.00 | 53.85 |
| 9 | Turmeric | 34.00 | 47.69 |
| 10 | Control | 65.00 | --- |
| | **S.Em.±** | **1.266** | |
| | **C.D. at 5%** | **3.735** | |
| | **C.V. (%)** | **11.05** | |

Raju and Naik (2006) found that nimbicidin (2.5 %) and garlic extracts (5 %) gave 100 and 67.46 per cent mycelial growth inhibition of blue mold rot (*Penicillium digitatum*) of onion, respectively.

Maharshi *et al.* (2009) reported that eucalyptus, garlic and henna extracts each at15 per cent gave 80, 60 and 54.00 per cent mycelial growth inhibition of *Penicillium italicum* causing blue mold rot of kinnow mandarin, respectively.

#### 4.5.2.2 *In vivo*

All nine phytoextracts were found significantly superior in reducing the Penicillium rot severity as compared to control on 4th and 8th day after inoculation both in pre- and post-inoculation treatments at 10 per cent conc. (Table 4.9 & Fig. 4.8).

##### 4.5.2.2.1 Pre-inoculation

The results presented in Table 4.9 and Fig. 4.8 revealed that the significantly lowest rot severity was recorded in fruits treated with tulsi, garlic and henna extracts on 4th day after inoculation (2.00, 2.24, 2.95 %) followed by cinnamon (3.57 %) and neem leaf extract (3.73 %). Lowest severity was found in tulsi leaf extract (3.00 %) and which was at par with garlic (3.68 %), and henna (4.70 %) extract, followed by cinnamon (7.39 %) and neem leaf extract (8.20 %) on 8th day after inoculation. Turmeric (14.30 & 28.66 %) proved least effective in managing the rot on 4th and 8th day after inoculation, respectively.

#### 4.5.2.2.2 Post-inoculation

Significantly lowest rot severity was recorded in tulsi leaf extract (2.90 %) and it was at par with garlic cloves extract (3.55 %) and henna leaf extract (4.50 %) on 4th day after inoculation. Tulsi (4.00 %) proved best in reducing the rot severity and it was at par with garlic (4.50 %) and henna (6.00 %) extract followed by cinnamom (8.53 %) and neem leaf extract (10.43 %) on 8th day after inoculation. Turmeric proved least effective in reducing the Penicillium rot severity (32.53 & 47.33 %) over control (40.00 & 96.50 %) on 4th and 8th day after inoculation, respectively.

The results of present findings are quite confirmative with the results reported by Kaur and Verma (2004). They noted that dry neem leaf extracts ($5\times10^4$ µg/ ml) found most effective in pre- (94.44 %) and post inoculation (50.0 %) in controlling green mold rot (*Penicillium digitatum*) of kinnow fruits. Khair and Hafez (2006) reported that the leaf extracts of eucalyptus and lemon grass (10 %) found effective in controlling the Penicillium rot in pre (3.33 %) and post inoculation (6.67 %) treatments in orange up to one week of storage, respectively. Singh and Sumbali (2003) noted that leaf extract of *Azadirachta indica* (10 %) was most effective in controlling the *Penicillium expansum* infecting apple fruits.

Maharshi *et al.* (2009) found *Eucalyptus amygdalina* extracts effective in checking the Penicillium fruit rot incidence (76.4 %) followed by *Allium sativum* (72.1 %), *Lawsonia inermis* (67.2 %) *and Curcuma longa* (60.8 %) each at 15 per cent over control in kinnow mandarin. Similar trend of results was observed in post inoculation treatment. Nath *et al.* (2012) reported that the *Ocimum sanctum* extract (50 %) effectively controlled Penicillium rot of kinnow mandarin fruit, followed by carbendazim (1000 ppm), *Azadirachta indica* (50%), *Aloe barbadensis* (50%) + carbendazim (1000 ppm) at 28 days during ambient storage.

**Table 4.9: Effect of Phytoextracts on the severity of Penicillium fruit rot of citrus *in vivo***

| Sr. No. | Phytoextracts (10%) | Rot severity (%) | | | |
|---|---|---|---|---|---|
| | | Pre-inoculation | | Post-inoculation | |
| | | 4th Day | 8th Day | 4th Day | 8thDay |
| 1 | Tulsi | 2.00 | 3.00 | 2.90 | 4.00 |
| 2 | Garlic | 2.24 | 3.68 | 3.55 | 4.50 |
| 3 | Henna | 2.95 | 4.70 | 4.50 | 6.00 |
| 4 | Cinnamom | 3.57 | 7.39 | 6.15 | 8.53 |
| 5 | Neem | 3.73 | 8.20 | 7.75 | 10.43 |
| 6 | Lemongrass | 6.33 | 16.80 | 14.53 | 19.73 |
| 7 | Aloevera | 8.43 | 20.33 | 19.39 | 25.63 |
| 8 | Eucalyptus | 12.93 | 25.66 | 30.35 | 41.13 |
| 9 | Turmeric | 14.30 | 28.66 | 32.53 | 47.33 |
| 10 | Control | 31.30 | 95.33 | 40.00 | 96.50 |
| | **S.Em.±** | **0869** | **0.57** | **1.14** | **1.358** |
| | **C.D. at 5%** | **3.37** | **1.68** | **3.37** | **4.00** |
| | **C.V. (%)** | **13.9** | **6.44** | **8.19** | **8.69** |

### 4.5.3 Antagonists

#### 4.5.3.1 *In vitro*

Five antagonists viz., *Trichoderma viride*, *T. harzianum*, *T. virens*, *Pseudomonas fluorescens* and *Bacillus subtilis* (Plate 4.11) were studied for their antagonism against *Penicillium citrinum* by dual culture method.

All the antagonists significantly helped in inhibiting the mycelial growth of *P. citrinum* over control. Significantly highest mycelial growth inhibition was observed in *T. harzianum* (77.41 %) followed by *P. fluorescens* ( 73.70 %). The next best treatment in the order of merit was *T. viride* (70.37 %) followed by *T. virens* (55.38 %). *Bacillus subtilis* showed minimum mycelial growth inhibition (22.59 %) after 8 days of incubation. (Table 4.10, Fig. 4.9 and Plate 4.12).

Result similar to the present investigation was reported by Chettri *et al.* (2005). They reported that native fluorescent *Pseudomonas* isolates found effective in inhibiting the growth of green mold *(Penicillium digitatum)* and blue mold *(Penicillium italicum)* pathogens infecting mandarin orange. *Pseudomonas fluorescens* found most effective in controlling the blue mould rot (*Penicillium expansum* or *Penicillium solitum*) of apple (Etebarin *et al.* 2005). Bagwan (2003) reported the potentiality and viability of *Trichoderma* spp. and *Candida* spp.

to control green (*Penicillium digitatum*) and blue mold (*Penicillium italicum*) rots of citrus, respectively.

**Table 4.10: Effect of antagonists on mycelial growth and per cent growth inhibition of *Penicillium citrinum in vitro***

| Sr. No. | Antagonist | Mycelial growth (mm) | Percent Growth Inhibition (PGI) |
|---|---|---|---|
| 1 | *Trichoderma viride* | 20.00 | 70.37 |
| 2 | *Trichoderma harzianum* | 15.25 | 77.41 |
| 3 | *Trichoderma virens* | 30.12 | 55.38 |
| 4 | *Pseudomonas flourescens* | 17.75 | 73.70 |
| 5 | *Bacillus subtillis* | 52.25 | 22.59 |
| 6 | Control | 67.5 | ---- |
| | **S.Em.±** | **0.52** | |
| | **C.D. at 5%** | **1.56** | |
| | **C.V. %** | **2.33** | |

### 4.5.3.2 *In vivo*

All the antagonists were found significantly superior in reducing the Penicillium fruit rot severity after 4th and 8th day of incubation in pre- and post-inoculation methods (Table 4.11 and Fig. 4.10)

#### 4.5.3.2.1 Pre-inoculation

*Trichoderma harzianum* was found significantly superior in reducing the Penicillium fruit rot severity by 4.50 and 10.00 per cent on 4th and 8th day after inoculation, respectively, followed by *P. flourescens* (6.50 & 13.50 %), *T. viride* (8.50 & 15.50 %) and *T. virens* (10.00 & 19.50 %) over control

(20.50 & 38.25 %). *Bacillus subtilis* found least effective in reducing the rot severity (14.50 & 24.50 %) (Table 4.10 & Fig. 4.9). Similar trend of results was recorded by Meena (2006). He found that *T. viride* and *T. harzianum* ($1\times10^6$ CFU/ml) could effectively control the Penicillium fruit rot (*P. fellutatanum*) of aonla. Bagwan (2003) recorded lowest Penicillium rot severity in citrus (*P. digitatum* and *P. italicum*) when the fruits were treated with *Candida oleophila, Candida* sp. and *T. viride*. Godara (1994) reported *T. harzianum* as most potent antagonist in inhibiting the growth of *Penicillium* sp. and *Botryodiplodia theobromae* infecting sweet oranges. Yadav (2009) recorded lowest Penicillium rot severity in aonla (*P. funiculosum* Thom.) when the fruits were treated with *T. harzianum.*

**Table 4.11: Effect of antagonists on severity of Penicillium fruit rot of citrus *in vivo***

| Sr. No. | Antagonist | Rot severity (%) Pre-inoculation | |
|---|---|---|---|
| | | 4th day | 8th day |
| 1 | *Trichoderma viride* | 08.50 | 15.50 |
| 2 | *Trichoderma harzianum* | 04.50 | 10.00 |
| 3 | *Trichoderma virens* | 10.00 | 19.50 |
| 4 | *Pseudomonas flourescens* | 06.50 | 13.50 |
| 5 | *Bacillus subtillis* | 14.50 | 24.50 |
| 6 | Control | 20.50 | 38.25 |
| | **S. Em. ±** | **00.65** | **01.13** |
| | **C. D. at 5%** | **01.95** | **03.35** |
| | **C.V. %** | **12.20** | **11.21** |

## 4.6 Effect of Hot Water Treatment on Penicillium rot of citrus *in vivo*

All the treatments found significantly superior and are at par with each other in reducing the Penicillium rot severity after 4th and 8th day of inoculation over control.

The fruits exposed to hot water treatment at all five treatments (Table 4.12 and Fig. 4.11) showed 100 per cent reduction in Penicillium rot incidence over control upto 8 days after inoculation at 25±1°C without any changes in natural fruit color.

The results revealed that the hot water treatment either at 50±2°C for 3 min. or 50±2°C for 4 min. found most effective in reducing the Penicillium rot severity in citrus fruits, without hampering the quality of fruits.

Results of present investigation corroborate with the results obtained by Palou *et al.* (2001). Valencia oranges when exposed to 50- 60 °C hot water reduced the Penicillium rot severity to 100 percent over control after 7 days of incubation. Inkha and Boonyakiat (2010) reported complete control of Penicillium rot in tangarin fruits, when treated with hot water at 50±2°C for 3 min. and 55±2°C for 2 and 3 min. upto 15 days. Smoot and Melvin (1963) found that immersion of oranges in hot water at 53°C successfully controlled green mold rot

(*Penicillium digitatum*). Houck (1967) reported that the hot water treatment to Furaka lemons at 52°C effectively controlled green mold (*Penicillium digitatum*) rot. Further Schirra and D'hallewin (1997) noted that the hot water treatment at 50-54°C did not injure the rind of Fortuna mandarins, but 56-58°C temperatures were found injurious to the fruits.

**Table 4.12: Effect of Hot Water Treatment on severity of Penicillium fruit rot of citrus *in vivo***

| Sr. No. | Temperature and min. | Rot severity (%) | |
|---|---|---|---|
| | | Post-inoculation | |
| | | 4th day | 8th day |
| 1 | 50±2°C, 3 min. | 00.00 | 00.00 |
| 2 | 50±2°C, 4 min. | 00.00 | 00.00 |
| 3 | 55±2°C, 2 min. | 00.00 | 00.00 |
| 4 | 55±2°C, 3 min. | 00.00 | 00.00 |
| 5 | 55±2°C, 4 min. | 00.00 | 00.00 |
| 6 | Control | 32.50 | 97.5 |
| | **S. Em. ±** | **0.33** | **0.26** |
| | **C. D. at 5%** | **099** | **0.78** |
| | **C.V. %** | **12.30** | **3.24** |

## 4.7 Effect of Hot Water Treatment on protein patterns (SDS-PAGE) of citrus fruit peel

Soluble proteins of citrus fruit peel from four different samples i.e. untreated healthy, fruits inoculated with of *P. citrinum* before treating with hot water at 50±2°C for 4 min.,

fruits inoculated with *P. citrinum* before treating with hot water at 55±2°C for 5 min. and fruits inoculated with *P. citrinum* without Hot water treatment were separated by SDS-PAGE on 10 % gel. The profile results showed the bands ranging from 30-35 with protein molecular weight ranging from 15 kDa to 195 kDa. The protein patterns of HWT samples at 25 kDa exhibited thicker band compared to inoculated untreated and uninoculated untreated heathly fruit peels.

The protein pattern of citrus fruit peels treated with HWT (50±2°C, 4 min. and 55±2°C, 4 min.) showed the presence of 103 kDa of molecular weight of proteins but these bands were absent in healthy and untreated inoculated protein samples (Plate 4.13).

The present findings of banding pattern of citrus fruit peels corroborate with the results of Inkha and Bonyakiat (2010). Protein patterns of tangerine fruit peels treated with HWT appeared to have 112.20 and 100KDa protein only on fifth day of storage due to heat stress circumstances in the fruit compared to untreated and uninoculated fruit peels.

The similar trend of results also reported by Pavaoncello (2001) in grape fruit of cv 'Star Ruby'.

Pavaoncello *et al.* (2001) showed that Hot water brushing treatment at 62 °C for 20 sec. against *Penicillium*

*digitatum* in Star Ruby induced the accumulation of the 21, 22 and 25 KDa chitinase protein and of 38 and 43 KDa β-1-3 glucanase proteins.

Chitinase, β-1-3 glucanase are considered as key enzymes having direct activity against pathogens in plant disease resistance system (Cao and Jiang, 2006)

Porat *et al.* (2001) showed using RNA gel blot hybridization that the suppression of the genes coding chitinase was markably induced both by hot water brushing and UV illumination.

Porat *et al.* (2002) showed using RNA gel blot hybridization that the suppression of the genes coding and β-1-3 glucanase was markably induced both by hot water brushing and UV illumination.

Mccollum *etal.* (1997) reported that the 22 KDa protein is one of the chitinase isoform that is normally abundant in heat stressed grape fruit.

# V. SUMMARY AND CONCLUSION

The present investigations on "Studies on Penicillium fruit rot (*Penicillium citrinum*) of citrus (*Citrus aurantifolia* Swingle) and its management" was carried out in the Department of Plant Pathology, B.A. College of Agriculture, Anand Agricultural University, Sardar Patel vegetable market and Goya Talav market, Anand. The results obtained on various aspects are summerised here under.

Freshly infected diseased citrus fruits (cv-Kagzi lime) showing typical symptoms of Penicillium rot were collected from Sardar Patel vegetable market, Anand. Penicillium rot first appears as water soaked lesion. The infected area enlarges and whitish mycelial growth appear on the surface of infected fruits followed by production of olive green spore mass.

The Koch's postulates of isolated pathogen were proved on healthy matured citrus fruits following wound inoculation method. All the inoculated fruits produced typical penicillium rot symptoms after four days of inoculation. The artificially inoculated fruits exhibited water soaked, light brown, soft areas easily punctured by finger pressure. The area enlarges in concentric circles with whitish mycelial growth producing light green to olive green spore mass in eight days. Reisolation from artificially inoculated diseased fruits produced *Penicillium* sp.,

which was found identical with original one. The reisolated pure culture of the pathogen was sent for identification to Indian Type Culture Collection (I.T.C.C.) Division of Mycology and Plant Pathology, I. A. R. I., New Delhi 110 012 and was identified as *Penicillium citrinum* (ID. No.8298.11).

On PDA, The fungus produced bluish green mycelial growth with abundant sporulation. In initial stage the fungal growth was white, rapidly becoming light green to olive green with production of conidia. The fungus produced whitish blue septate mycelial growth. The conidophores are branched twice or thrice, elongate, bearing long tangled chaines of conidia.

The weekly survey carried out from first week of October to fourth week of March, 2010-11 revealed that the incidence of Penicillium rot was predominant (6.48 & 5.95 %) followed by bacterial canker (4.79 & 4.74 %) and Aspergillus rot (3.88 & 3.78 %) at Sardar Patel vegetable market, station road and Goya Talav market, Anand, respectively.

The activities of polygalaturonase and polymethylgalacturonase enzymes were studied on fruits inoculated with *Penicillium citrinum* along with healthy fruit. The activity of PG was higher in inoculated fruits (33.32 %) than healthy fruits (9.29 %). No significant difference was observed in the activity of PMG between inoculated and healthy fruit peel.

The RAPD analysis was performed among five isolates of *Penicillium citrinum* collected from AND, VDA, ABD, DKR and NAV. RAPD analysis of the DNA of five isolates with 15 primers showed amplification of total 160 bands, with a range of 16 to 7 bands per primer. Overall, *Penicillium* isolates exhibited a moderate level of genetic diversity. The maximum 16 bands were generated by a single primer (primer 0PA- 12); whereas, primer 0PA-11, OPE-14, OPE-15, OPE-3 and OPA-9 generated 15, 14, 14, 12 and 11 bands respectively. The lowest number of bands was generated by primer OPA-1 and OPA-10 (7 bands). Dendrogram (Figure 4.4) based on "Nei's (1978) unbiased measures of genetic distance by UPGMA method" formed two clusters. Cluster 1 includes four isolates *viz.*, AND, ABD, DKR and NAV. While cluster 2 includes a single isolate i.e. VDA. The similarity coefficient ranged from 0.42 to 0.53 with all the 15 primers. The maximum genetic similarity was found between the isolate AND and ABD which are included in cluster 1. Whereas, the least Genetic similarity was found between the isolate VDA and AND which are included in two different clusters (1 & 2). Primer OPA-13, OPA-10, OPE-17 and OPE-18 showed 100 per cent polymorphism, followed by OPA-9 (90.90 %), OPE-11 (88.88 %), OPA-1 and OPE-15 (85.71 %) per cent polymorphism of the DNA which helped to ascertain variability among the isolates of *P. citrinum*.

Out of nine fungicides screened *in vitro*, seven fungicides i.e. Azoxystrobin and azoxystrobin 18.2 % + difenconazole (250 and 500 ppm), mancozeb (1000 and 2000 ppm), hexaconazole (5 %) + captan (70 %), carbendazim (12 %) + mancozeb (63 %), cymoxanil (8 %) + mancozeb (64 %), carbendazim (500 and 1000 ppm) were found significantly superior in inhibiting mycelial growth of *P. citrinum* (100 %) over control. While hexaconazole and thiophanate methyl (500 and 1000 ppm) gave mediocre effect in inhibiting the mycelial growth (20.45 %, 22.34 % and 17.92 %, 19.92 %) at 500 and 1000 ppm, respectively.

Mancozeb at 2000 ppm ( 2.70 %), and carbendazim, at 500 and 1000 ppm (4.19 & 2.90 %) proved significantly superior over all other treatments both in pre and post-inoculation treatments respectively after 8 days of inoculation. The higher conc. of mancozeb (2000 ppm) found best for reducing the Penicillium rot severity both in pre- and post-inoculation treatments. Thiophante methyl and hexaconazole found least effective in controlling the rot.

The nine phytoextracts (10 %) were screened to know their efficacy in controlling the Penicillium fruit rot of citrus *in vitro* and *in vivo*. *In vitro*, all nine phytoextracts screened were found significantly superior in inhibiting the mycelial growth of

*P. citinum* over control. Tulsi, garlic and henna extracts completely inhibited the mycelial growth (100 %) over control. The next best treatment in order of merit were cinnamon (9.00 mm) and neem (12.30 mm) showing 86.15 and 81.80 per cent growth inhibition, respectively over control (65 mm). Tulsi leaf extract proved most effective in reducing the Penicillium fruit rot severity both in pre- (3.00 %) and post-inoculation (4.00 %) treatments and it was at par with garlic (3.68 & 4.50 %) and henna (4.70 & 6.00 %) after 8 days of incubation. The pre-inoculation treatment is more effective than post-inoculation.

Five antagonists i.e. *Trichoderma viride*, *T. harzianum, T. virens, Pseudomonas flourescens and Bacillus subtilis* were screened *in vitro* against *P. citrinum* by dual culture technique. The highest mycelial growth inhibition of *P. citrinum* was recorded in *T. harzianum* (77.41 %) followed by *Pseudomonas flourescens* (73.70 %). *In vivo* condition, *T. harzianum* (10.00 %) was found most potent antagonist in reducing the severity of Penicillium rot followed by *P. flourescens* (13.50 %) in pre-inoculation treatment, after 8 days of incubation.

The fruits exposed to hot water treatment at all five treatments showed 100 per cent reduction in Penicillium rot incidence over control upto eight days after inoculation at 25±1°C without any changes in natural fruit color. Hot Water

treatment either at 50±2°C for 3 min. or 50±2°C for 4 min. found most effective in reducing the Penicillium rot severity in citrus fruits, without hampering the quality of fruits.

The SDS-PAGE protein profile results showed the number of bands ranging from 30-35 with protein molecular weight of 15 KDa to 195 KDa. The protein pattern of citrus fruit peels treated with HWT showed the presence of 103 KDa molecular weight of proteins but this band was absent in healthy and inoculated untreated samples. The protein patterns of HWT samples at 25 kDa exhibited thicker band compared to inoculated untreated and uninoculated untreated heathly fruit peels.

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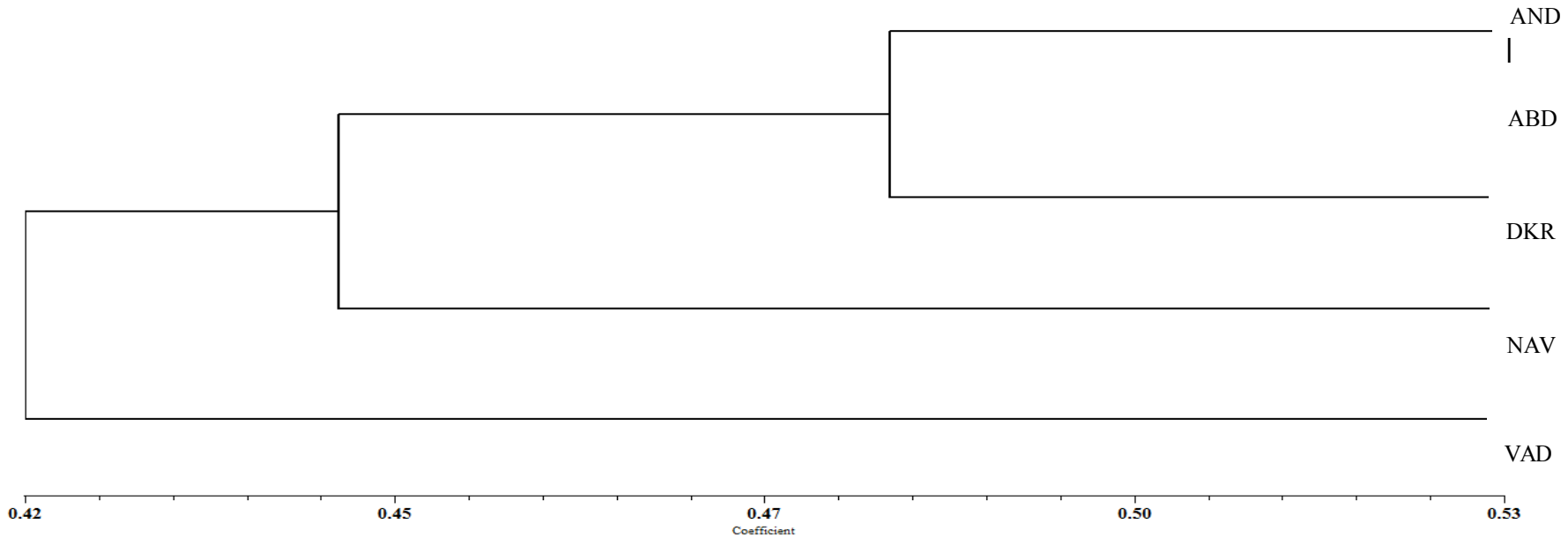



**Fig. 4.4: Dendrogram of five isolates of *Penicillium* based on Nei's (1978) similarity coefficient using UPGMA as the clustering method for RAPD**

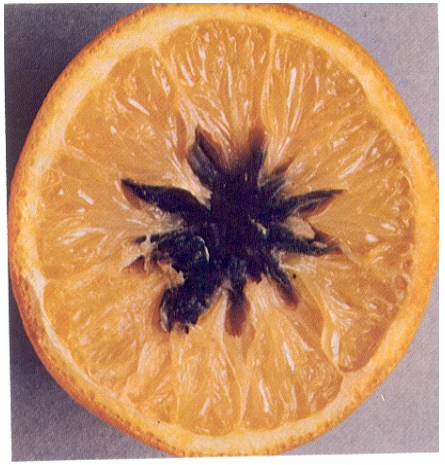
Citrus canker

Aspergillus rot

Stem-end rot

Penicillium rot

Phytophthora rot



Alternaria rot

**Plate 4.5: Post-harvest diseases of citrus**

**0%**

**1-20%**

**21-40%**

**41-60%** **61-80%** **>80%**

**Plate 3.1: Assessment key used to record the severity of Penicillium fruit rot of citrus.**

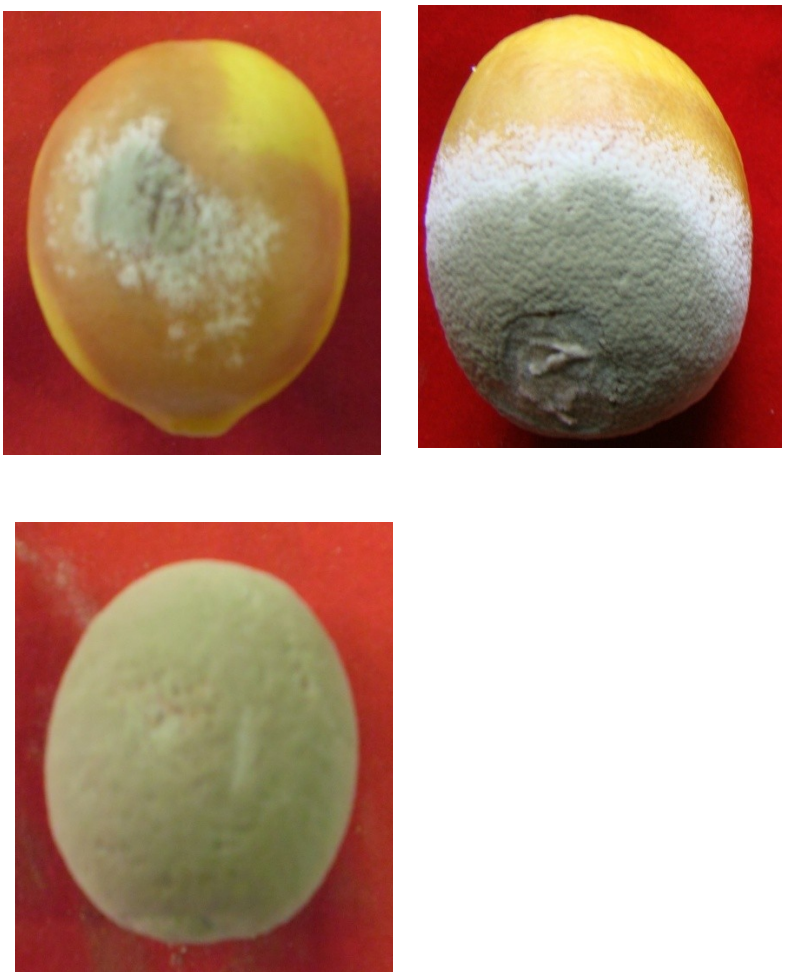
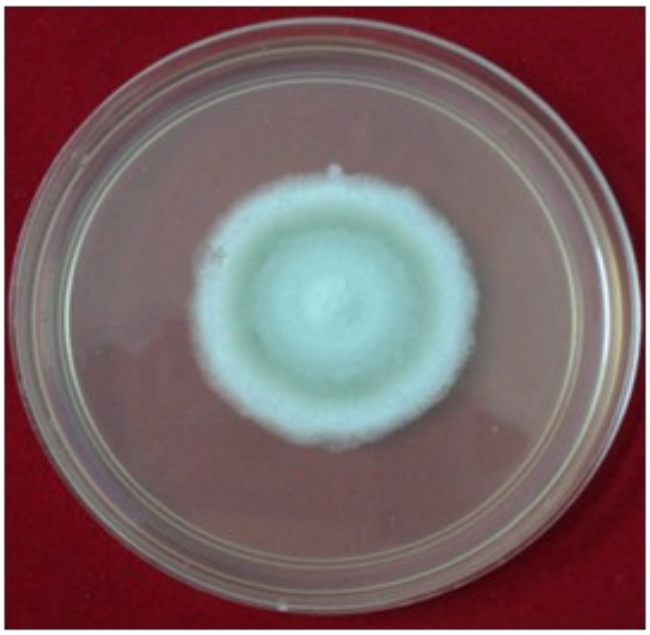


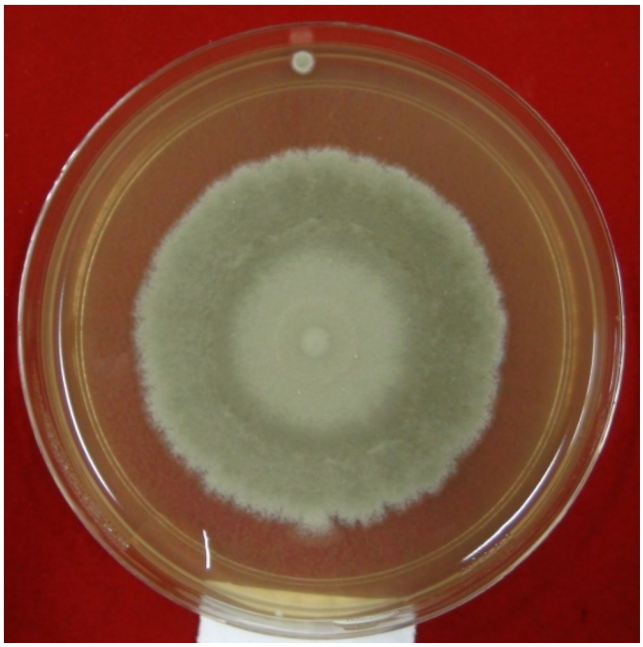
**Plate 4.1 Typical symptoms of Penicillium fruit rot of citrus**

**Uninoculated** **Inoculated**

**Plate 4.2: Pathogenicity test of *Penicillium citrinum***



4 days after inoculation



7 days after inoculation

**Plate 4.3: Mycelial growth of *Penicillium citrinum***

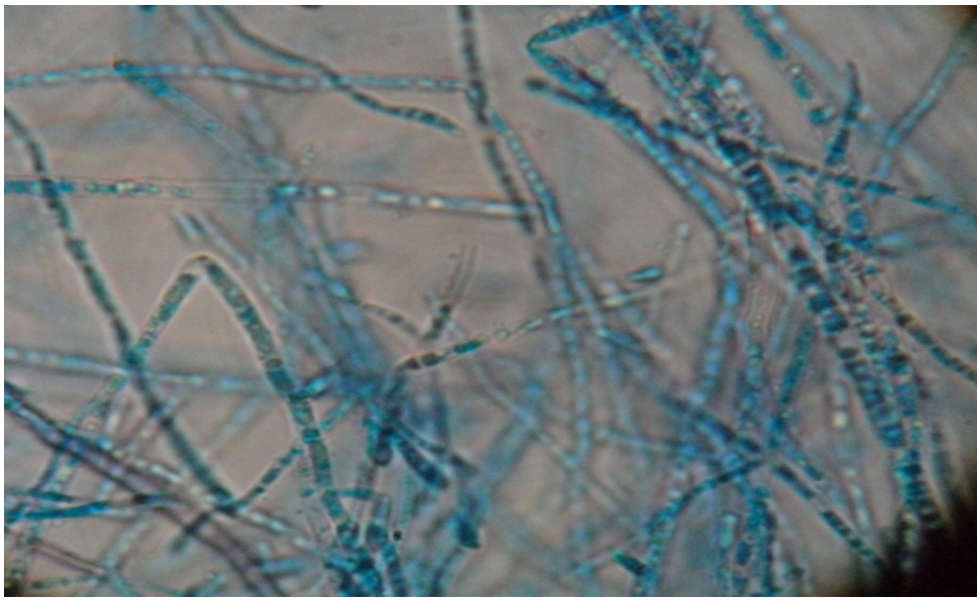


**Plate 4.4 (a): Microphotograph of mycelium of *P. citrinum***

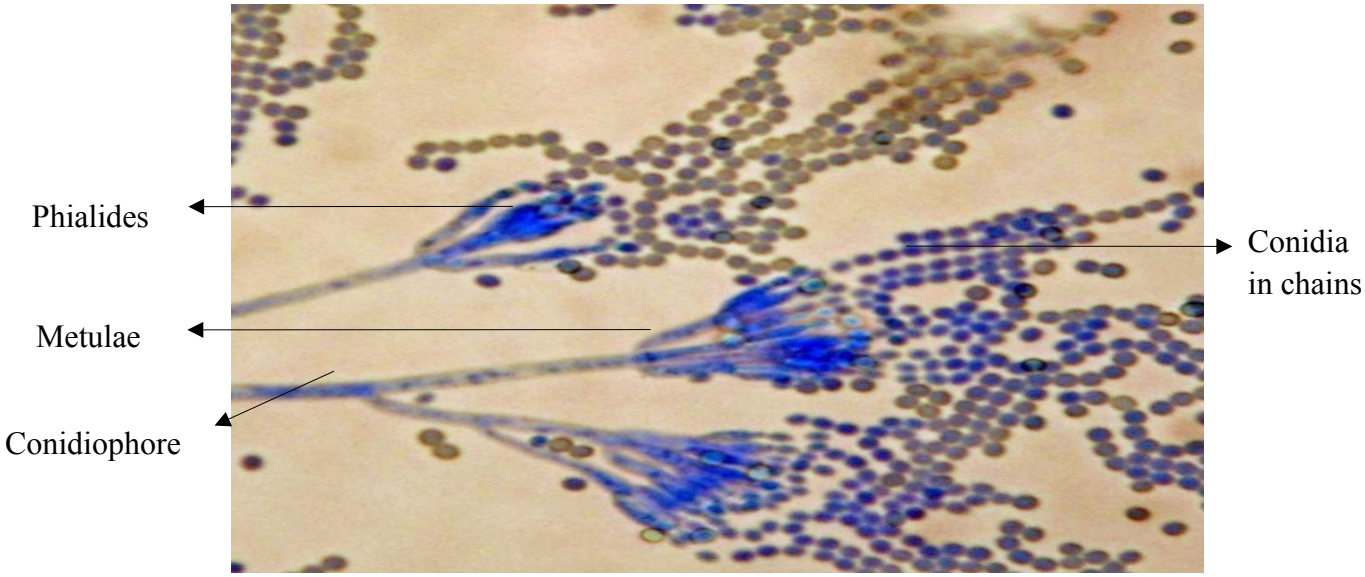
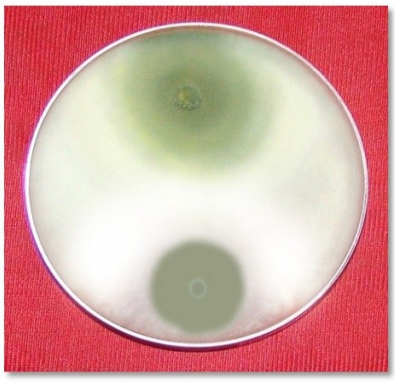
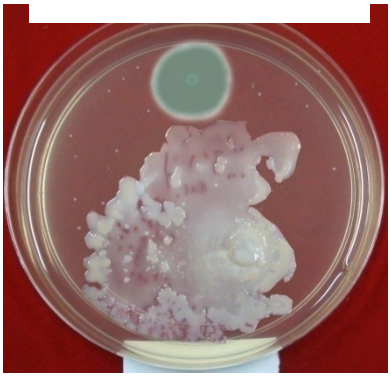



**Plate 4.4 (b): Microphotograph of spores of *P. citrinum***

*T. harzianum*

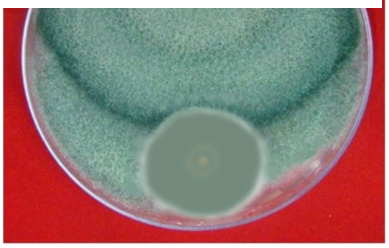


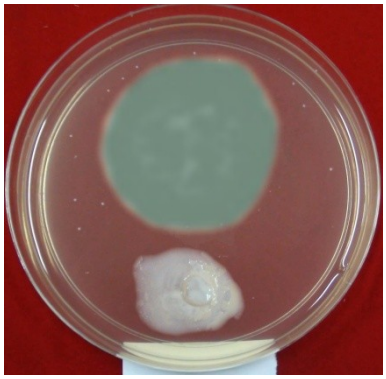
*T. Viride*



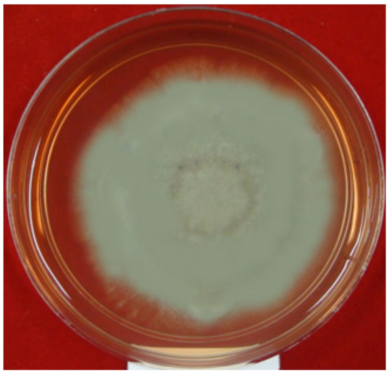
*P. flourescens*

*T. virens*





*B. subtilis*



Control

**Plate 4.12: Effect of antagonists against *Penicillium citrinum in vitro***

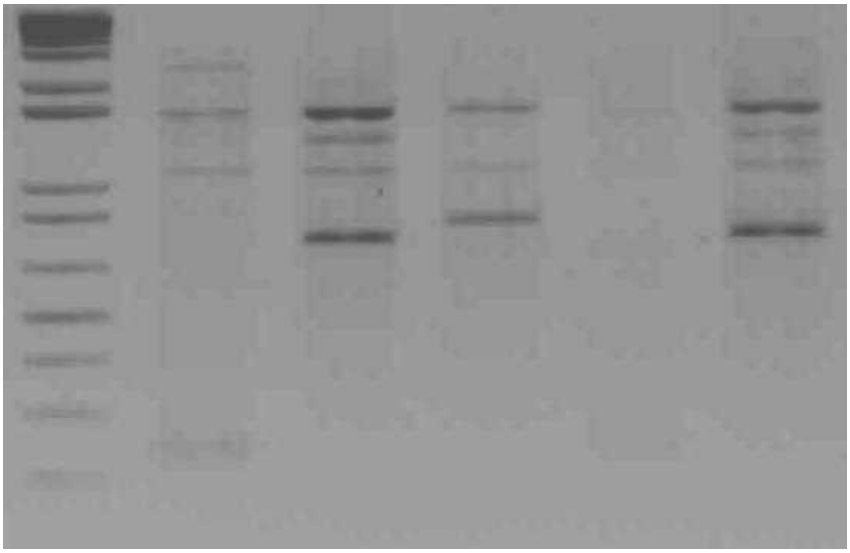


**Plate 4.6 : RAPD profile OPA-1**

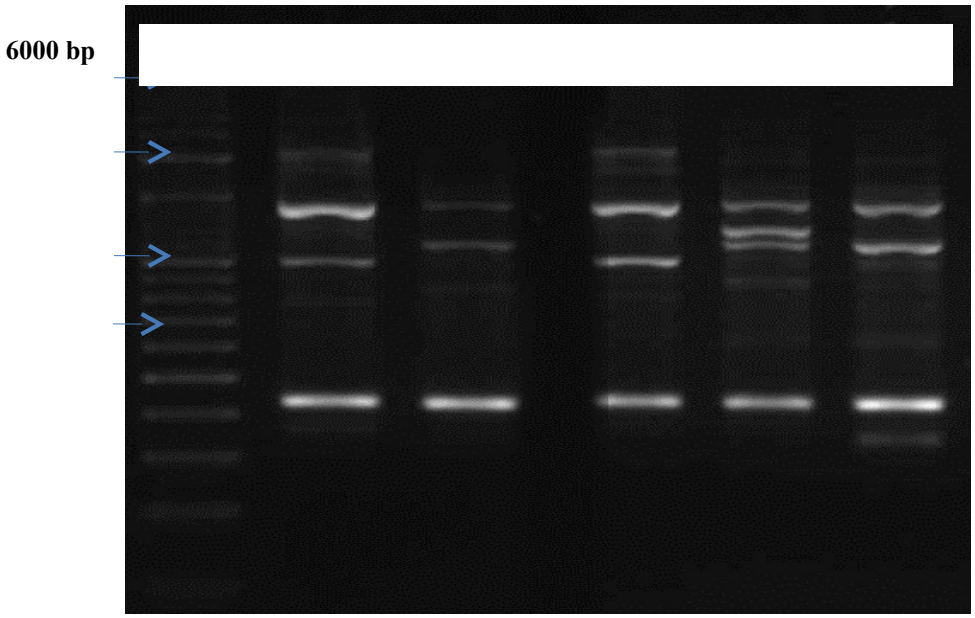



**Plate 4.6: RAPD profile OPA-5**

**M = 100 + 500 kbp DNA ladder; 1=AND; 2=VDA; 3=ABD; 4=DKR; 5=NAV**

**Molecular characterization of *Penicillium citrinum* isolates by RAPD Marker**

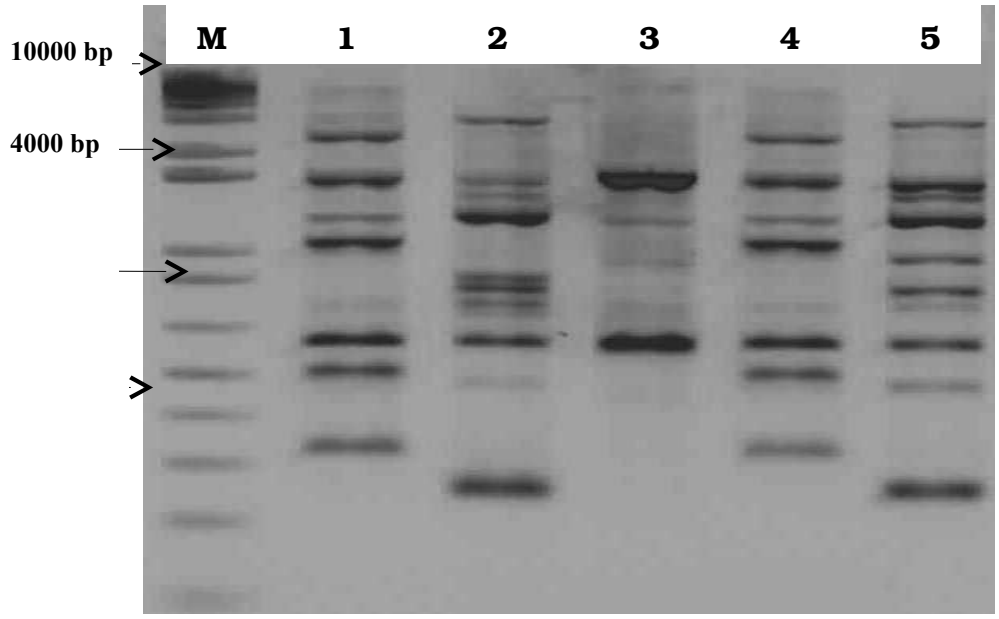



**Plate 4.6: RAPD profile OPA-11**

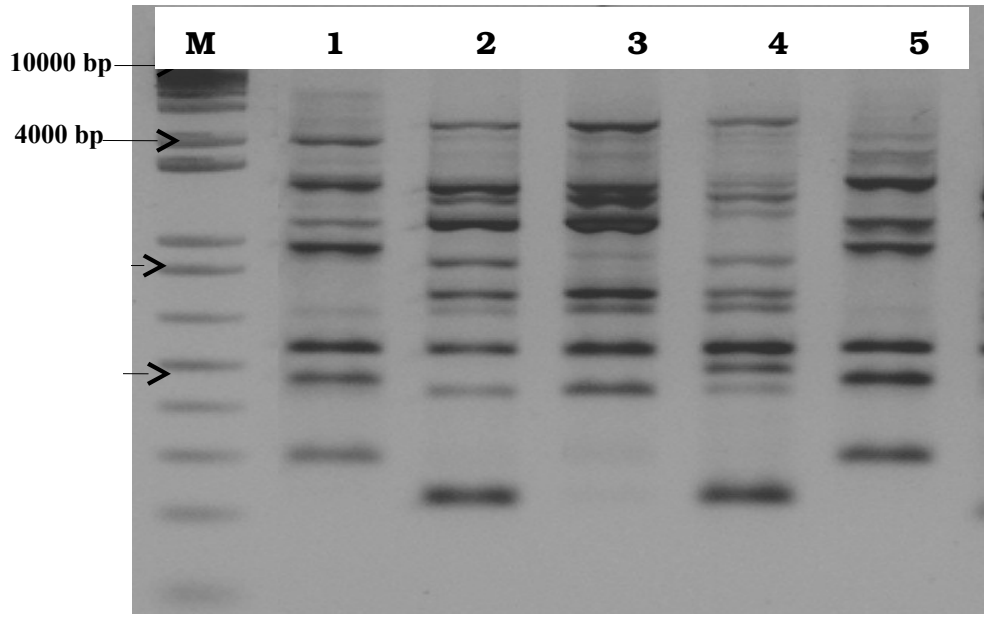



**Plate 4.6: RAPD profile OPA-12**

**M = 100 + 500 kbp DNA ladder; 1=AND; 2=VDA; 3=ABD; 4=DKR; 5=NAV**

**Molecular characterization of *Penicillium citrinum* isolates by RAPD Marker**

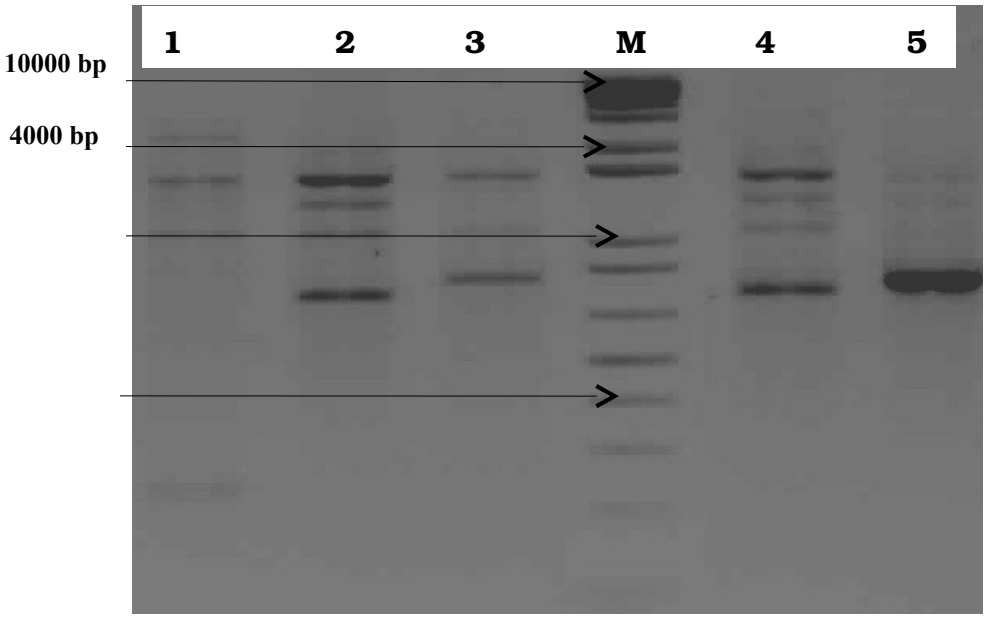



**Plate 4.6: RAPD profile OPA-13**

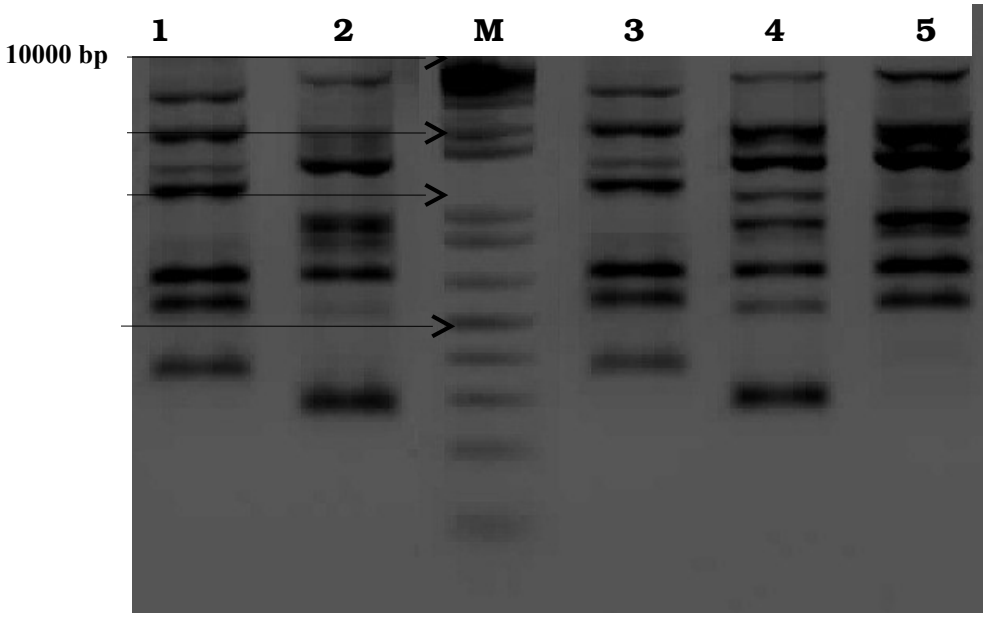



**Plate 4.6: RAPD profile OPA-18**

**M = 100 + 500 kbp DNA ladder; 1=AND; 2=VDA; 3=ABD; 4=DKR; 5=NAV**

## Molecular characterization of *Penicillium citrinum* isolates by RAPD Marker

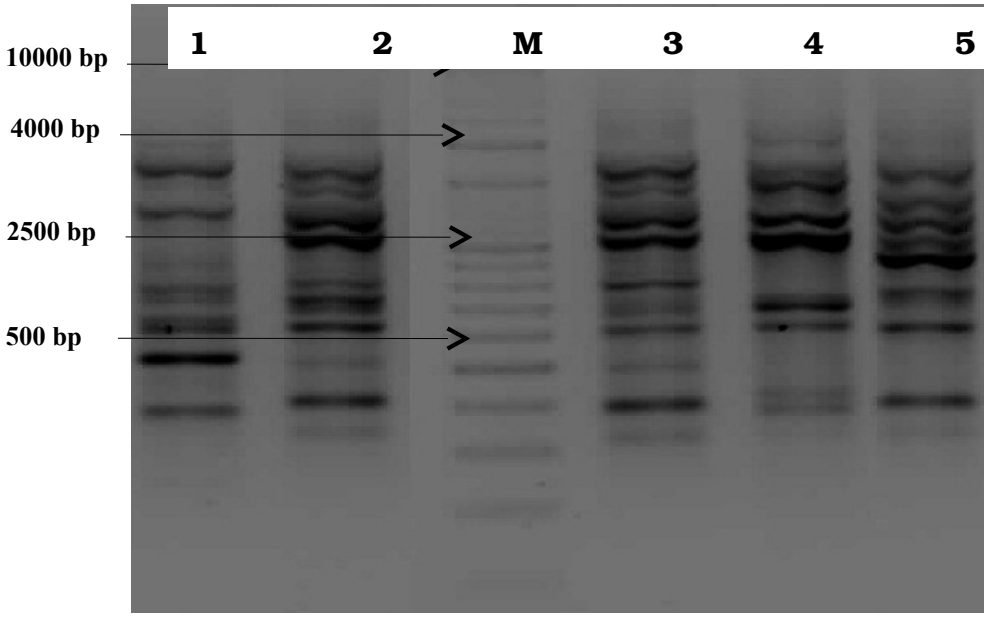



**Plate 4.7: RAPD profile OPE-3**

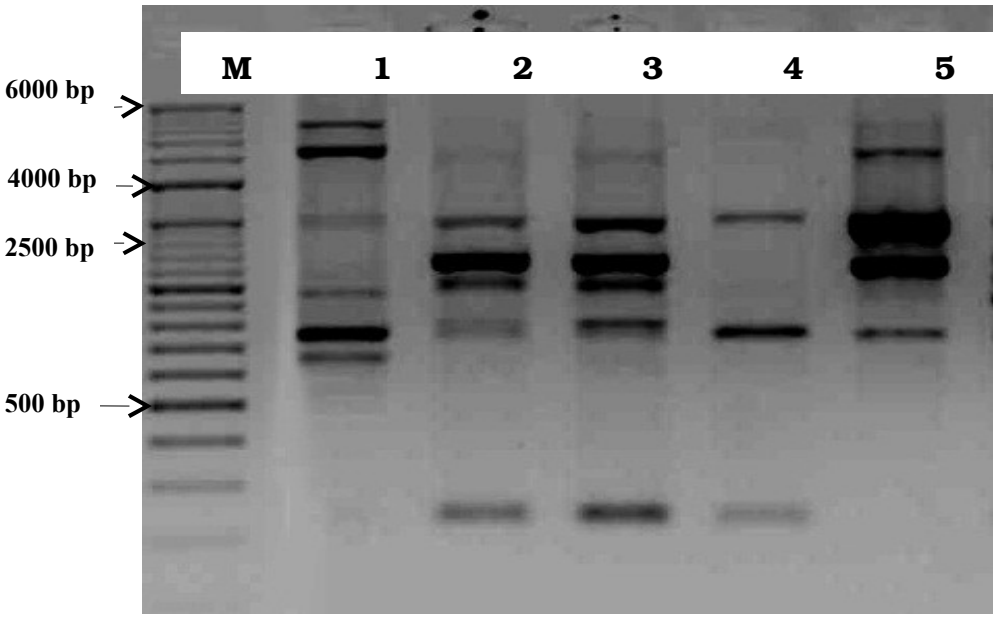



**Plate 4.7: RAPD profile OPE-11**

**M = 100 + 500 kbp DNA ladder; 1=AND; 2=VDA; 3=ABD; 4=DKR; 5=NAV**

## Molecular characterization of *Penicillium citrinum* isolates by RAPD Marker

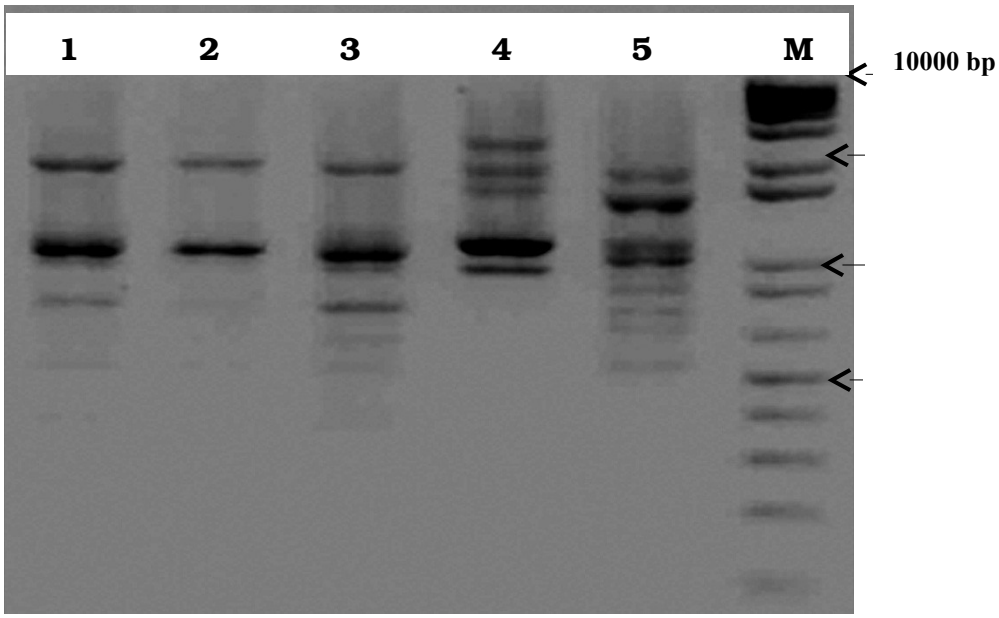



**Plate 4.7: RAPD profile OPE-12**

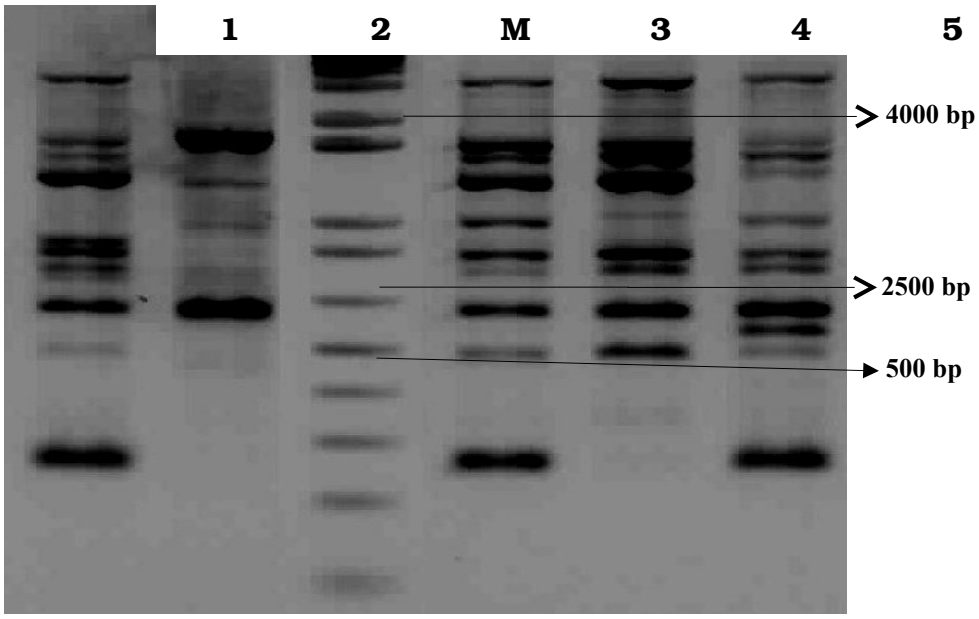



**Plate 4.7: RAPD profile OPE-14**

**M = 100 + 500 kbp DNA ladder; 1=AND; 2=VDA; 3=ABD; 4=DKR; 5=NAV**

**Molecular characterization of *Penicillium citrinum* isolates by RAPD Marker**

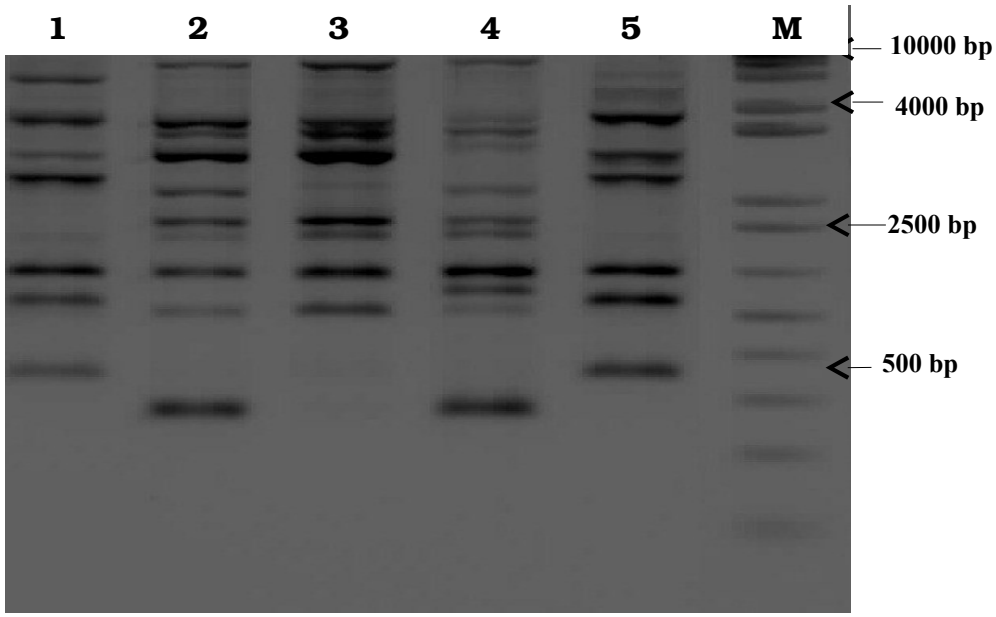



**Plate 4.7: RAPD profile OPE-15**

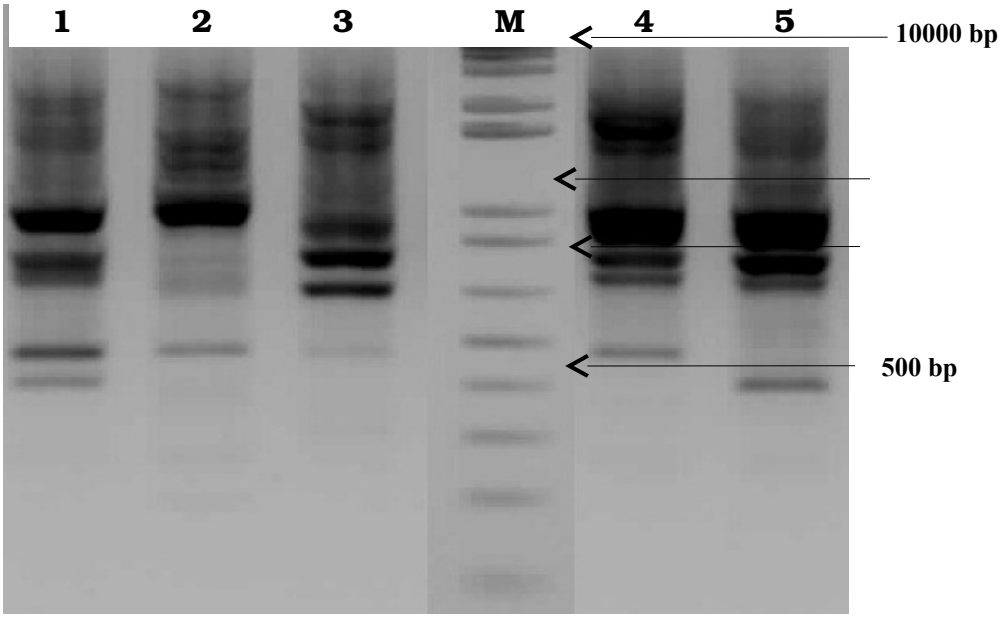



**Plate 4.7: RAPD profile OPE-17**

**M = 100 + 500 kbp DNA ladder; 1=AND; 2=VDA; 3=ABD; 4=DKR; 5=NAV**

**Molecular characterization of *Penicillium citrinum* isolates by RAPD Marker**

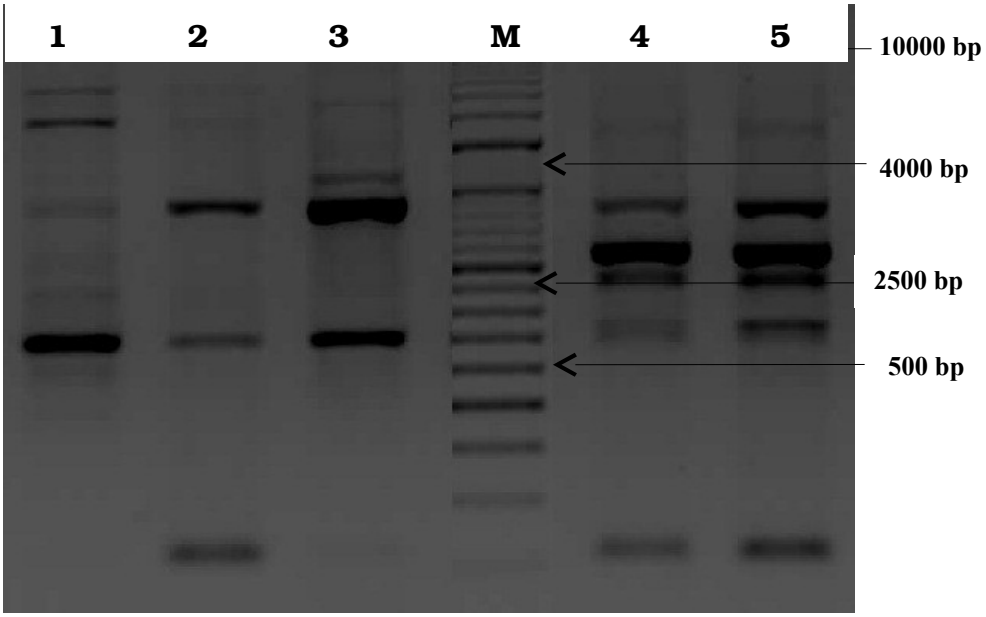



**Plate 4.7: RAPD profile OPE-18**

**M = 100 + 500 kbp DNA ladder; 1=AND; 2=VDA; 3=ABD; 4=DKR; 5=NAV**

**Molecular characterization of *Penicillium citrinum* isolates by RAPD Marker**

M= Protein Molecular Marker 29 to 200 kDa

1= Fruit inoculated with *P. citrinum* and treated with HW (50±1ºC for 4min)

2= Fruit inoculated with *P. citrinum* and treated with HW (55±1ºC for 4min)

3= untreated healthy fruit

4= Fruit inoculated with *P. citrinum* with out HWT

***P. flourescens***