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Morphological, pathogenic and genetic variability in castor wilt isolates

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ABSTRACT: Castor wilt caused by *Fusarium oxysporum* f.sp. *ricini* is an economically important and major disease of castor. Variation in resistance to wilt was observed among castor hybrids/ varieties in different cultivated areas because of pathotypes. Isolates of fungus were collected from different castor growing areas of India and morphological, cultural characters were studied. The effect of culture filtrates of these isolates was tested on germination of castor seeds and wilt incidence with a view to study differences in the toxin production among the isolates. The isolates showed variation in mycelial growth, pigmentation, sporulation, size of conidia and production of micro and macroconidia. Culture filtrate of the isolates reduced the germination of castor seeds. The germination was directly correlated with the concentration of the culture filtrate in comparison to check. Maximum inhibition in germination was observed with undiluted culture filtrate followed by 100 and 75 ppm concentration. The isolates also differed in the degree of inhibiting the root growth. All the seedlings wilted in undiluted culture filtrate of all isolates whereas, no wilting was observed in seedlings kept at 25 ppm conc. Isolates varied in production of toxin and thereby wilt incidence. The reaction of the 29 isolates on castor cultivars indicated the existence of different virulence of isolates in different castor growing areas of country and five pathotypes of the pathogen were characterized. Isolates 2,14 and 15 were virulent and attacked DCS 9 and Kranthi cultivars. The genetic variation of isolates of *F. oxysporum* f.sp. *ricini* was studied by random amplified polymorphic DNA analysis. The isolates were grouped into five clusters based on molecular polymorphism generated by RAPD primers. The grouping of isolates based on pathogenic variation has no correlation with grouping based on RAPD analysis with most of the test isolates.

Key words: Castor wilt, *Fusarium oxysporum* f.sp. *ricini*, isolates, morphological, pathogenic variability, RAPD analysis

Castor is one of the important non-edible oilseed crop extensively grown in India mainly as a rainfed crop in *kharif* season primarily in the states of Gujarat, Andhra Pradesh and Rajasthan besides other states on a limited scale. Wilt caused by *Fusarium oxysporum* f.sp. *ricini* is a major disease occurring in many castor growing areas of the country (Kolte,1995). This disease was recorded in India from Udaipur (Rajasthan) in 1974 (Nanda and Prasad, 1974) and the disease incidence was observed to the extent of 20% on Gujarat hybrid. The extent of yield loss depends on the stage at which the plants are affected, 77% at flowering stage, 63% at 90 days and 39% at later stages on secondary branches (Pushpavathi, 1995). The major strategy to manage the disease is to deploy wilt resistant varieties/hybrids. Host plant resistance as a tactics of disease management can be highly effective looking into the soil-borne nature of the pathogen. However, this approach has been hampered by the appearance of races which make the resistant varieties/hybrids susceptible (Katan *et al.*, 1994). Variability is the very basis of survival of the pathogen and in order to cope up with the host diversity and fluctuating weather, it produces different races. There is a wide diversity in the variability of the pathogen as characterized by reaction on the host differentials. Little work has been done on the pathogenic variability of *F. oxysporum* f.sp. *ricini.* Hence, studies were undertaken to determine the comparative morphological and pathogenic variability

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among the isolates of *F. oxysporum* f.sp. *ricini* and to study the genetic variation of these isolates by random amplified polymorphic DNA analysis.

MATERIALS AND METHODS

Pathogen isolates

Wilt affected plant samples were collected from different castor growing areas of the country. *F. oxysporum* f.sp. *ricini* (*For*) was isolated from these samples and single spores of each isolate were transferred to potato dextrose agar (PDA) medium and incubated at 25±1°C. Twenty nine isolates of *For*, fourteen isolates from Andhra Pradesh; 1 to 14 (Rajendranagar, Sankarpalli, Kandukur, Kothaguda, Palem, Mahaboobnagar); fourteen isolates from Gujarat; 15 to 28 (Ahmedabad, SK Nagar, Jallundhari, Talod, Baroda, Rathanpuri, Soyla, Junagadh) and one isolate 29 from Tamil Nadu (Yethapur) were used during the course of study.

Cultural and morphological variation

Mycelial bits of 5 mm diameter was removed from 7-day-old culture of each isolate, inoculated on PDA and incubated at 25 ± 2°C for 7 days. The morphological and cultural characteristics of colony were assessed for mycelial growth, pigmentation, sporulation, size of micro and macroconidia. Thirty conidia under different microscopic fields were considered for taking measurements of length and width.

Effect of culture filtrate of different isolates on germination and root length of castor

The isolates were grown separately in Modified Richard's solution in 250 ml conical flasks for 2 weeks at 25 ± 2°C. The mycelial growth of the isolates was filtered through Whatman's filter paper to obtain pure culture filtrate. Different concentrations of culture filtrate viz., 25, 50, 75, 100 ppm and pure/undiluted culture filtrate (UCF) were prepared. Castor seeds of (susceptible cv. Kranthi) were soaked in culture filtrate of different concentrations for overnight and the following day they were placed in equidistance in petri plates with two layers of filter paper moistened with 5 ml of 25, 50, 75 and 100 ppm along with undiluted culture filtrate of each isolate in triplicate. For each concentration thirty seeds were used. A check was maintained with sterile distilled water for comparison. The petriplates were incubated at 25 ± 2°C for 7 days. The number of seeds germinated out of total seeds was recorded and percentage germination was calculated. Root length of germinated seeds was measured.

Effect of culture filtrate of various isolates on wilt incidence of castor seedlings

Different concentrations of culture filtrate (25, 50, 75, 100ppm and undiluted culture filtrate) were prepared. Castor seeds of cv. Kranthi were sown in sterilized sand. Ten days old seedlings were removed from sand, washed with tap water then with distilled water and kept in culture filtrate of different concentrations. The seedlings kept in distilled water served as check. The seedlings were observed for the wilt symptoms and the percentage of seedlings wilted was recorded.

Standardization of inoculation technique for pathogenic variation studies

Three methods of inoculation *viz.*, root dip, soil drenching with inoculum and soil infestation were conducted with five isolates. In root dip inoculation, 10 day old seedlings were removed from sand, the roots were trimmed at the ends and dipped in spore suspension of 1×10^6 conidia / ml for 90 seconds and transplanted in pots containing sterilized soil.

In soil drenching method, seedlings of 15-20 days old were used. Spore suspension (1×10^6 conidia / ml) was poured around the root zone in the soil by disturbing the root zone.

In soil infestation method, the fungus was grown on autoclaved sorghum seeds for 10-15 days. The inoculum was mixed with soil and left for 7- 10 days. Then castor seeds were sown in the infested soil.

Pathogenic variability

Pathogenicity test was conducted for the isolates on susceptible cv. (VP-1) by root dip inoculation technique. Pathogenic variability studies were conducted by using four castor cultivars, DCS 89, DCS 9, 48-1 (resistant) and Kranthi (susceptible). Root dip inoculation technique was

followed (Raoof and Nageswara Rao, 1996). The seeds were soaked in captan solution (0.2%) for 2 hours and sown in plastic trays containing sterilized sand.

The isolates of the fungus were grown on sterilized sorghum seeds for 7 days. The inoculum was prepared by transferring few infected sorghum seeds into sterile water and shaken thoroughly using a magnetic stirrer. The concentration of spore suspension was adjusted to 1 x 10^6 spores/ml with haemocytometer and root dip inoculation was followed as described above. Seedlings dipped in sterile distilled water served as control. Each pot contained four seedlings and each cultivar was replicated twice. The seedlings were observed for the development of disease and wilt incidence was recorded as number of plants wilted out of total plants inoculated, up to 60 days after transplanting.

Genetic variation studies

DNA isolation: The genomic DNA of each isolate was extracted by the standard CTAB method of Lee and Taylor (1990). The cultures were grown in potato dextrose broth (PDB) for 4 days under shaking conditions at 25 ± 2°C. The mycelium was collected on sterile whatman no.1 filter paper, blotted dry and ground to fine powder in liquid nitrogen using prechilled mortar and pestle.

RAPD analysis: The RAPD reaction was performed as described by William *et al.* (1990) using random primers (Operon tech Inc,.CA). PCR was carried out in 20 µl of reaction mix containing 5.0ng DNA, 0.8 µl dNTPs, 2 µl of 10x PCR buffer, 2 µl of primer, 0.4 µl Taq polymerase. Amplification was performed in a thermal cycler programmed for one cycle of 3 minutes at 94°C, 45 cycles of 20 sec at 92°C, 30 sec at 36°C and one minute at 72°C and a final extension at 72°C for 2 min. After PCR, the amplified products were electrophoresed on 1.8 % agarose gels containing ethidium bromide and visualised under UV transilluminator. Initially, 50 random primers from 26 sets (OPA to OPZ) were screened with 4 geographically different isolates. Of these, adequate polymorphism was observed with primers from OPL set. Hence, 20 primers from Kit OPL of Operon Technologies Inc. (OPL 1 to OPL 20) were used. The data was analysed by a phenogram based on the Jaccard coefficient of similarity that was computed by the unweighed pair group method using arithmetic averages (UPGMA).

RESULTS AND DISCUSSION

Morphological and cultural variation

The isolates showed great variability in colony characteristics such as mycelial growth, pigmentation, sporulation, size of conidia and production of micro and macro conidia. Mycelial growth varied from profuse raised, fluffy to thin and flat. Pigmentation varied from light pink to dark pink, violet to dark violet, rosy purple to dark purple and orange.

In isolates *For* 11, 21, 26, 27 and 28 the mycelial growth was thin, flat white mycelium with dark violet, rosy purple and dark pinkish violet pigmentation, respectively. The mycelial growth was profuse, raised, fluffy, woolly white mycelium with violet to dark violet pigmentation in isolates *For* 1, 2, 3, 5, 8, 15, 17, 22, 25, 29 and light rosy purple to dark purple pigmentation in isolates *For* 4, 9, 14, 16, 18, 19, 20, 23 and 24. Isolate *For* 5 produced concentric rings in the medium. Profuse, raised pinkish mycelium with light to dark pink pigmentation was observed in *For* 6, 10, 12 and 13 while isolate *For* 7 produced profuse, raised with light orange woolly mycelium with dark orange pigmentation. Nanda and Prasad (1974) observed typical white fluffy mycelial growth of *F. oxysporum* f.sp. *ricini* with pinkish pigmentation. Desai *et al.* (1994) suggested that growth variability was useful in distinguishing 4 races of *F. oxysporum* f.sp. *ciceris* which causes chickpea wilt. Okiror and Kimani (1997) reported strong difference in growth traits such as growth rate, growth habit and morphology in isolates of *F. udum* of pigeon pea wilt fungus.

Sporulation ranged from scanty to very high. Sporulation was scanty in *For* 3, 6, 18, 20, 21, 23 and 27. Isolates *For* 4, 11, 25 and 29 yielded moderate sporulation. Spore production was high in *For* 5, 7, 9, 10, 12, 16, 17, 19, 26 and 28. Isolates *For* 1,2, 8, 13, 14, 15, 22 and 24 had shown very high sporulation in the medium. Desai *et al.* (2003) reported differences in cultural and morphological characteristics of 15 isolates of *F.oxysporum* f.sp.*ricini*. Although variability was observed in the cultural and morphological characters of the isolates,

some features were common to all the isolates, namely abundant production of microconidia and formation of both terminal and intercalary chlamydospores. Das and Sengupta (1998) reported similar results with regard to *F. udum*, pigeon pea wilt fungus. However, the proportion of macroconidia to microconidia varied in different isolates. Macroconidia were few and many microconidia observed in few isolates. In some isolates, both macro and microconidia were more. Macroconidia were 2 to 7 septate, straight to curve, sickle shaped or linear to broad. The average size of macroconidia ranged from 23.2 x 4.1 µm in *For* 22 to 64.5 x 5.4 µm in *For* 29. Microconidia were hyaline, round to oval in shape ranged from 9.5 x 3.2 µm in *For* 22 to 23.4 x 6.8 µm in *For* 19.

Effect of various concentrations of culture filtrate of different isolates on germination and root length of castor seedlings

The germination of castor seed was reduced in culture filtrate of all isolates. There was no germination in undiluted culture filtrate of isolates *For* 13, 14, 15 and 100 ppm concentration of *For* 28 (Fig.1) which indicates that these isolates produced high toxin concentration. Germination was cent percent in 25 ppm conc. of isolate *For* 21. In isolates *For* 1, 2, 5, 8, 9, 13, 14, 15, 19, 24 and 28 the fungus produced more toxin which reduced the germination drastically. With increase in concentration of culture filtrate of isolates *For* 2, 5, 8, 9 and 28, there was a decrease in germination. The germination was more in UCF of isolates *For* 17, 18, 21, 23 and 25.

Culture filtrate of 29 isolates of fungus showed reduction in root length. The root inhibition was more in *For* 1, 2, 13, 14, 15, 19, 21, 22, 23, 24 and 28. Maximum root inhibition occurred in undiluted culture filtrate as compared to other dilutions of culture filtrate. Isolates varied in the degree of inhibiting the root growth. Research work has not been done with culture filtrate of *F. oxysporum* f.sp. *ricini* but this type of differential behaviour of culture filtrate was reported with other pathogens like *Colletotrichum falcatum*, red rot fungus in sugarcane (Chandrika *et al.,*1982) and *Sclerotium oryzae*, stem rot fungus in rice (Ali and Singh, 1992). Culture filtrate of 15 isolates of *S. oryzae* inhibited the radicle and plumule elongation in rice that was directly correlated with the concentration of the culture filtrate.

Effect of different concentrations of culture filtrate of various isolates on wilt incidence

Castor seedlings kept in undiluted culture filtrate of all isolates were killed, as the toxin concentration was more. Isolates *For* 2, 14, 15, 17, 24 and 28 caused complete wilting of all seedlings kept at 75 ppm and 100 ppm concentration (Fig. 2), which

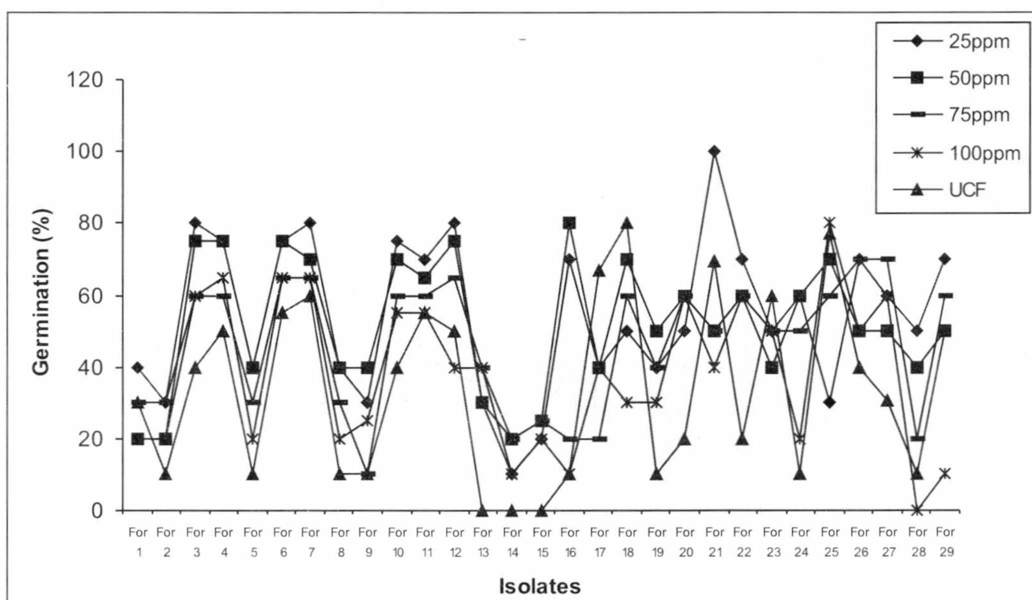

Fig. 1. Effect of different concentrations of culture filtrate of various isolates of For on germination

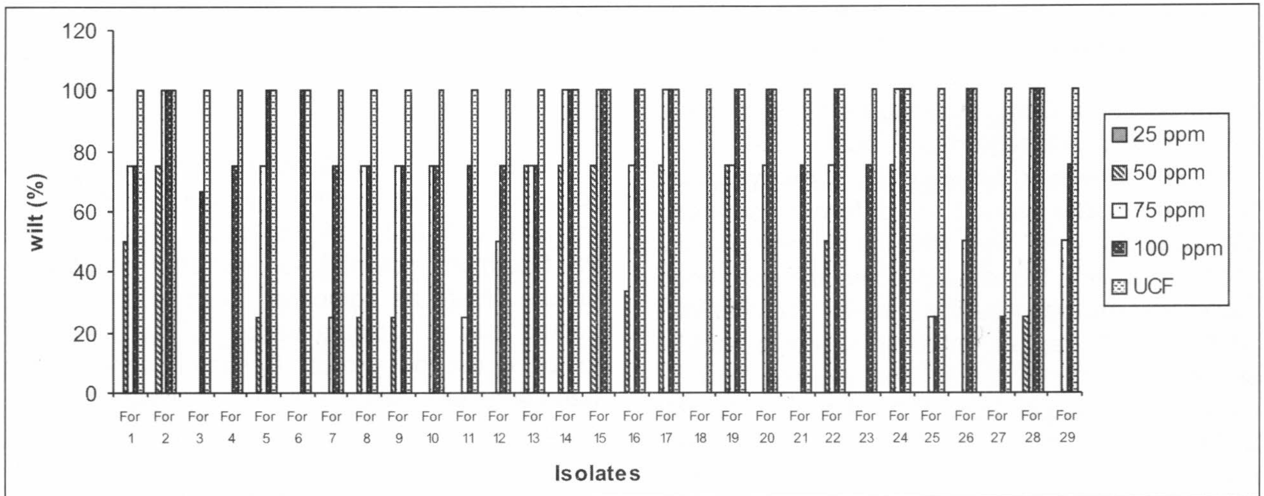

Fig. 2. Effect of different concentrations of culture filtrate of different isolates on wilt incidence

indicates that these isolates are highly virulent and produced more toxin. Isolates *For* 1, 5, 8, 9, 10, 13, 16, 19, 20 and 22 showed 75% wilting of castor seedlings at 75 ppm conc. of culture filtrate. Isolates *For* 2, 13, 14, 15, 17, 19 and 24 caused 75% wilting of seedlings even at 50 ppm concentration of culture filtrate and these isolates could cause wilting even at low concentration of toxin. In isolates *For* 3, 4, 6, 21, 23 and 27 the seedlings were not wilted at 25, 50, 75 ppm conc. of culture filtrate and this may be due low virulence. Isolate *For* 18 is less virulent, as seedlings were fresh and not wilted at all concs. of culture filtrate tested except undiluted culture filtrate. Wilting was not observed in seedlings kept in 25 ppm concentration of all 29 isolates may be because of low toxin concentration.

Standardization of inoculation technique for pathogenic variation: Among the three methods of inoculation, root dip inoculation and soil drenching were superior in showing wilt symptoms on inoculated plants. In root dip inoculation method, the wilt symptoms appeared within a week after inoculation, whereas in soil drenching method, symptoms appeared 25-30 days after inoculation. By root dip method, both isolates *For* 14 and 15 recorded 100 % wilt incidence. *For* 15 showed more wilt incidence in all inoculation methods (Fig.3). Raoof and Nageswara Rao (1996) reported that root dip inoculation technique was the simple screening technique for evaluation of large number of castor germplasm lines against castor wilt and this method can be repetitive. Desai and Dange

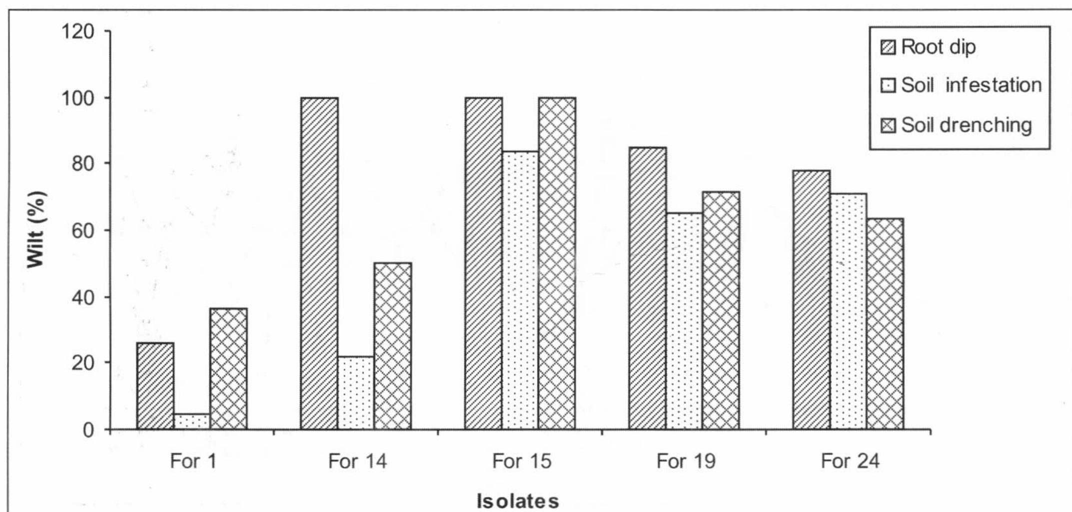

Fig. 3. Comparison of three methods of inoculation of wilt

(2003) showed that inoculum concentration of 1x10^6 spores/ml induced cent percent wilt incidence when clipped root system of 10 day old castor seedlings were dipped for 1-3 min.

Pathogenic variability studies: Significant difference in virulence on four castor cultivars was observed in different isolates. DCS-89 and 48-1 exhibited resistance to all 29 isolates. Isolates *For* 2, 14 and 15 were highly virulent and caused wilting in DCS 9, Kranthi and showed resistance to 48-1 and DCS-89 (Table 1). These isolates also caused complete wilting of seedlings of Kranthi and reduced germination of castor seeds at 50, 75, 100 ppm conc. and undiluted culture filtrate. These isolates belong to group I. Isolates *For* 1 and 24 showed resistant reaction with 48-1 and DCS 89, moderately resistant to DCS 9 and induced susceptible reaction in Kranthi. These isolates also caused wilting of seedlings of Kranthi at 50, 75, 100 ppm conc. and UCF. They are moderately virulent and belong to group II.

Isolates *For* 5, 8, 9, 16, 17, 19 and 28 constitute group III and they showed susceptible reaction in Kranthi and resistant to 48-1, DCS 89 and DCS 9. Isolates *For* 7, 11, 13, 18, 20, 22, 23, 26 and 29 belong to group IV, recorded resistant reaction to 48-1, DCS 89, DCS 9 and moderately resistant to Kranthi. The group V consisted of *For* 3, 4, 6, 10, 12, 21, 25 and 27 which were less virulent and showed resistant reaction in all cultivars tested. In isolates *For* 3, 4, 6, 21 and 27 culture filtrate of 100 ppm and UCF only caused wilting of seedlings of Kranthi and at 50, 75 ppm concentration there was no wilting of seedlings which may be due to low toxicity. Isolates *For* 1, 2 were isolated from wilted plants of hybrid GCH-4 a resistant hybrid which became susceptible to wilt in the recent 3 - 4 years, so they are comparatively virulent isolates than *For* 3 and 4.

Isolates 2, 14 and 15 are virulent isolates collected from different places, but they are similar in pathogenicity hence the virulence may not be dependent on the area of collection and may be because of genetic make up of isolates. Desai *et al.* (2003) reported that six out of 15 isolates of *F.oxysporum* f.sp.*ricini* collected from different areas of Gujarat proved highly virulent against VP-1 and VI-9. Several isolates of *F.udum* showed consistent differences in virulence on twelve pigeon pea lines

Table 1. Reaction of castor cultivars for different isolates of *F. oxysporum* f.sp. *ricini*

Isolates	48-1	DCS-89	DCS-9	Kranthi
For 1	R	R	MR	S
For 2	R	R	S	S
For 3	R	R	R	R
For 4	R	R	R	R
For 5	R	R	R	S
For 6	R	R	R	R
For 7	R	R	R	MR
For 8	R	R	R	S
For 9	R	R	R	S
For 10	R	R	R	R
For 11	R	R	R	MR
For 12	R	R	R	R
For 13	R	R	R	MR
For 14	R	R	S	S
For 15	R	R	S	S
For 16	R	R	R	S
For 17	R	R	R	S
For 18	R	R	R	MR
For 19	R	R	R	S
For 20	R	R	R	MR
For 21	R	R	R	R
For 22	R	R	R	MR
For 23	R	R	R	MR
For 24	R	R	MR	S
For 25	R	R	R	R
For 26	R	R	R	MR
For 27	R	R	R	R
For 28	R	R	R	S
For 29	R	R	R	MR

R = resistant (0 - 20% wilt incidence); MR = moderately resistant (20 - 49% wilt incidence); S = susceptible (> 50% wilt incidence).

(Okiror and Kimani, 1997). Zuniga *et al.*(1997) reported 2 races of *F. oxysporum* f.sp. *melonis* out of 46 isolates based on pathogenicity on a set of differential melon cultivars.

Isolates *For* 1, 2, 14, 15 and 24 were comparatively more virulent and they produced more sporulation (+ + + + +) in the medium. Similarly, Desai *et al.* (2003) also suggested that highly virulent isolates produced abundant sporulation and less mycelial growth while less virulent isolates produced poor sporulation and

more mycelial growth. Champawat and Pathak (1989) with *F. oxysporum* f.sp. *cumini* and Desai *et al.,* (2003) with *F.o.* f.sp. *ricini* reported similar type of results.

Genetic variation studies

DNA finger print profiles were developed for all the isolates. The isolates showed varied number of DNA bands (2-12) with each of the 20 primers employed (Fig.4). Data on DNA polymorphisms suggested that *F. oxysporum* f.sp. *ricini* isolates from different geographic origins form five distinct subpopulations. *For* 7 and 9 isolates had highest genetic diversity than the other isolates and formed cluster I. *For* 10 and 12 were closely related to each other in terms of DNA similarity and also in pathogenic variability and formed the II cluster. In the same way, isolates *For* 1 and 24 were relatively close to each other in genetic make up and pathogenic variability. Isolates *For* 2, 3, 4, 8, 5, 6, 11 and 15 were related to each other in genetic make up and constitute III group. Castor hybrid GCH 4 has been resistant to wilt for some time and it became susceptible only in 3-4 years. Isolate *For* 2 collected from infected GCH 4 was highly virulent isolate compared to other isolates. Woo *et al.* (1996) indicated wide genetic diversity in *F.o.* f.sp. *phaseoli* by studying different isolates by RAPD with 4 different primers and RFLP with 5 different restriction enzymes. RAPD markers were quick and reliable alternative for differentiating isolates of *F.o.* f.sp. *vasinfectum* into three cluster groups (Assigbetse *et al.*,1994). RAPD procedure is technically simple, rapid, requires only small amounts of DNA and involves no radioactivity (Manulis *et al.,* 1994).

IV cluster group consists of isolates *For* 26, 27 and 28,which were closely related to each other. Cluster group V consists of large number of isolates like *For* 1, 16, 24, 29, 18, 20, 22, 13,14, 17, 21, 23,

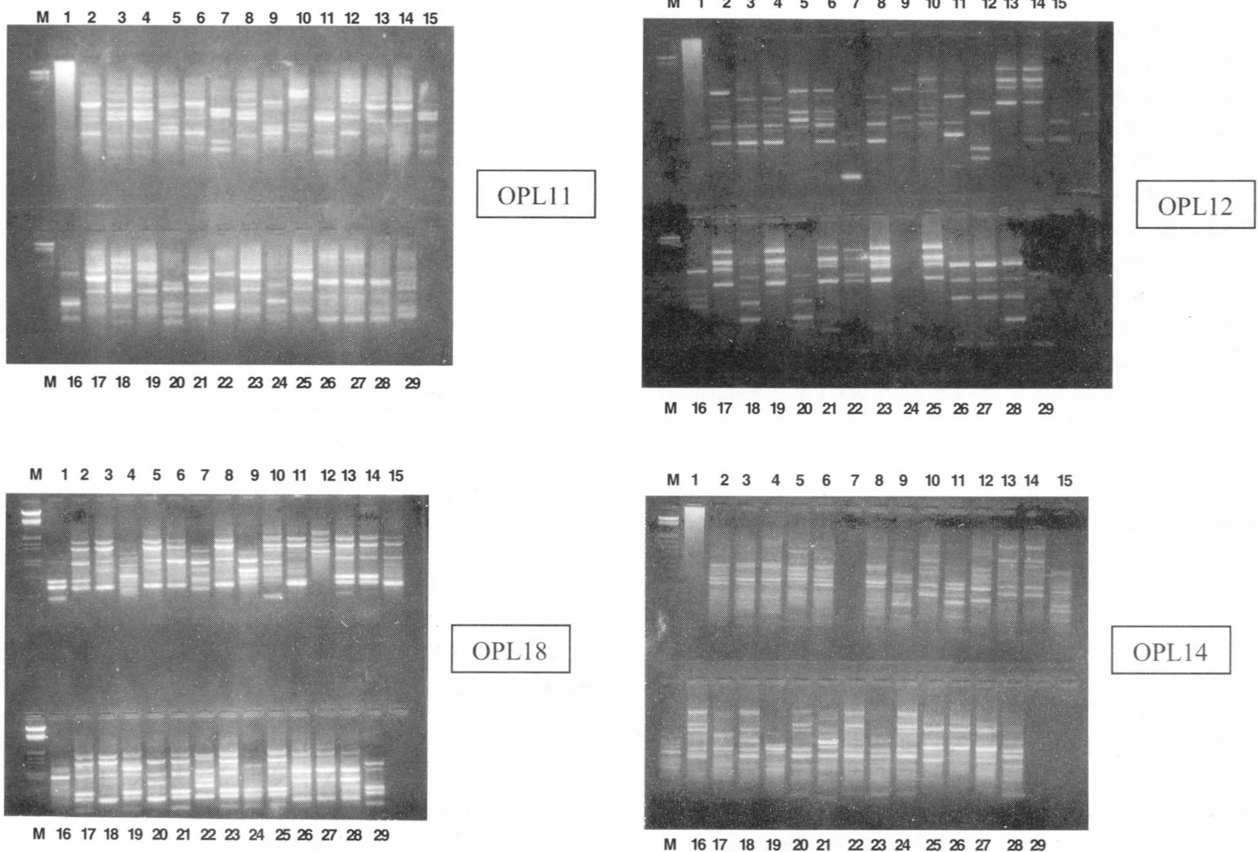

Fig. 4. RAPD banding patterns of isolates of *F.o.*f.sp.*ricini* with different OPL primers No.1-14: isolates from Andhra Pradesh; 15-28: isolates from Gujarat 29: isolate from Tamil Nadu; M- Marker.

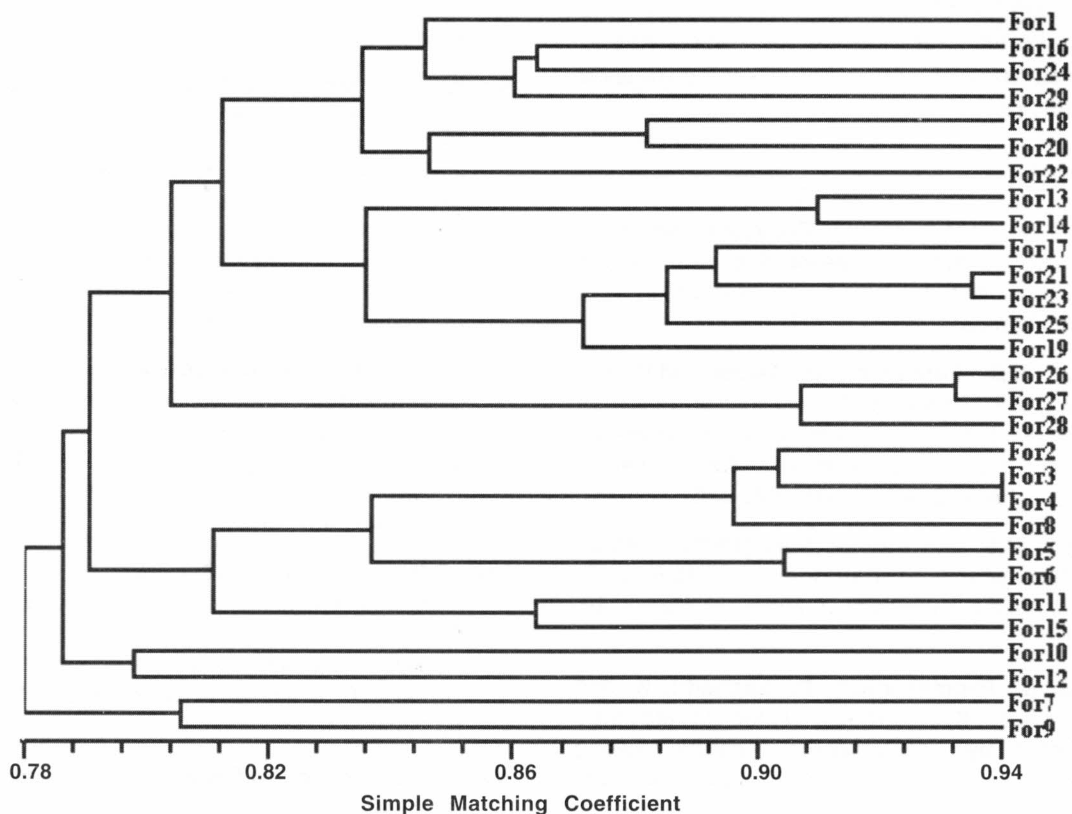

Fig. 5. Dendrogram representing genetic relationship among isolates of *Fusarium oxysporum* f.sp. *ricini*

25 and 19 which has similar type of genetic make up (Fig.5). Jimenez-Gasco *et al.* (2001) reported the presence of three clusters in 60 isolates of *F.o.* f.sp. *ciceris* by RAPD analysis. Isolates of *F.oxysporum* obtained from cucumber worldwide were classified into 3 groups by RAPD-PCR (Vakalounakis and Fragkiadakis, 2000). Dubey *et al.* (2005) identified presence of 7 races based on pathogenic variability and seven clusters based on RAPD analysis out of 25 isolates of *F.o.* f.sp. *ciceris.*

Isolates *For* 13 and 14 collected from same area showed similar genetic make up. This was true with *For* 2, 8, 3, 4, 5 and 6 collected from same area of AP. Isolates *For* 10 and 12 collected from same area of AP were similar in pathogenic and genetic studies. Same in the case of *For* 18, 20 and 22 collected from near by areas of Gujarat were similar in pathogenic and genetic studies, however the isolates *For* 1 and 2 collected from same area differed in pathogenic and genetic studies. This gives the inference that some isolates are similar in both pathogenic and genetic groups. However, some isolates like *For* 13, 14 (AP) 17, 21, 23, 25, 19 (Gujarat) collected from different areas have similar genetic make up but they differed in pathogenic variability. This indicates that grouping based on RAPD data could not be correlated to the groups based on morphology and pathogenicity in some isolates. Similar results were reported by Latha *et al.* (2002) in case of *Colletotrichum graminicola* of sorghum. Sivaramakrishnan *et al.*(2002) also reported that clustering of isolates of *F.udum* into different groups by RAPD and amplification fragment length polymorphism (AFLP) did not reflect the geographical distribution of the various pigeon pea growing areas from where the isolates were collected.

The results imply that the isolates have to be evaluated based on their genetic relation ships and differential host reaction rather than on a regional basis or location of collection. The studies indicate that there is variation among 29 isolates in terms

of morphological characters, pathogenicity and genetic make up. More number of isolates has to be collected from different areas of castor growing states and has to be studied.

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