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CHARACTERIZATION AND RELATIVE CONTRIBUTION OF FUNGAL AND BACTERIAL PATHOGENS INVOLVED IN SUDDEN DEATH SYNDROME OF CHILLIES

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A B S T R A C T

In the present study, bacterial and fungal agents involved in sudden death syndrome (SDS) in chillies and their relative contribution in the development of disease complex were investigated. Out of eight microbial agents isolated from wilted chilli plants, three pathogenic species viz. *Ralstonia solanacearum*, *Fusarium oxysporum* and *Rhizoctonia solani* were identified and characterized depending upon their morphological, cultural and biochemical behavior. These isolates induced wilting, when inoculated on susceptible chilli cultivar California Wonder under greenhouse conditions. All the pathogens induced wilt symptoms; however, the wilting was more rapid in case of *R. solanacearum* followed by *F. oxysporum* and *R. solani.* Disease symptoms were more prominent when three pathogens inoculated in combinations. However, *R. solanacearum* and *R. solani* intriguingly, showed no symptoms even after thirty days of inoculation upon repeated trials. On the other hand, the interactive studies of three pathogens indicated that the symptoms development and seedlings mortality were higher than the rest of combinations or by the individual pathogen.

Keywords: *Ralstonia solanacearum*, *Fusarium oxysporum, Rhizoctonia solani,* Chilli.

INTRODUCTION

The genus *Capsicum* belongs to the family *Solanaceae*. Out of about 20-27 species, only five species (*Capsicum annuum, C. chinense*, *C. frutescens, C. baccatum* and *C. pubescens*) are domesticated and cultivated in several areas of the world (Tong and Bosland, 1999; Bosland and Vaotava, 2000). In Pakistan, two species of *Capsicum* are common viz. *C. frutescens* Linn (sweet pepper) and *C. annuum* Linn (hot pepper) but most cultivated species is *C. annuum.* In Pakistan, chilies are grown over an area of 62.7 thousand hectares with the total production of 150.3 thousand tons having an average yield of 2.7 tons/ha (GOP, 2013). Average production of 2.7 tons/ha is far below than the potential yield of other chilli growing countries of the world. The inferior quality of the fruit and low per unit area production of chili is due to several biotic factors including plant diseases which are the major constraints in its production and productivity. Among them Sudden Death Syndrome (SDS) is the most important and serious one induced by number of soil borne wilt causing pathogens that act together in complexes rather than a specific disease. Sudden death syndrome (SDS) is a name given to a complex of different diseases that eventually leads to quick and rapid death of plants. Many pathogens are primarily involved in causing SDS in chilli. Severely affected plants die quickly and may leads towards the development of epidemic in the coming years. The symptoms were incited by a number of pathogens including the nematode such as *Meloidogyne incognita, Phytophthora capsici, Verticillium dahliae*, *F. oxysporum, Rhizoctonia solanacearum* and *R. solani.* Synergistic association among all wilt causing plant pathogens and nematodes might be expressed by an increase vascular wilt severity and ultimate death of the whole plant (Rather *et al*. 2012). *Ralstonia solanacearum* is a causal agent of bacterial wilt disease of chilli (Tahat and Sijam, 2010). The pathogen alone or in combination with fungal pathogens like *Phytophthora* spp. can cause huge yield

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losses in areas of vegetable production (Burney and Ahmed, 1997). The effect of *R. solanacearum* and *Meloidogyne* spp. is augmented with *Fusarium*s pp. which leads to the development of wilt complex disease (Deberdt *et al*. 1999). Similarly, the interactive effect of *R. solanacearum*, *F. oxysporum* and *R. solani* resulted in sudden wilting of the plants (Ammajamma 2010). The complex of *V. dahliae, P. capsici, R. solanacearum* and *F. oxysporum* were also found to be associated with the wilt syndrome of solanaceous crops (Begum *et al*. 2012). The disease complex involving *P. capsici, F. oxysporum* and *M. incognita* led to the development of syndrome disease in all major pepper growing countries of the world (Ramana and Eapen, 2000). The association of *Fusarium* species with *R. solani* or root knot nematodes *(Meloidogyne* spp.) caused significant yield losses in chili plants in Sindh Pakistan (Ehteshamul-Haque and Ghaffar, 1994). The wilt complexes was also hastened by co-infection *P. capsici* and *V. dahliae* the presence of these pathogens increase the wilt incidence and enhances the infection of chili plant (Sanogo, 2007).

Recently, this complex disease is becoming severe and conferring serious hazard to pepper production in Pakistan. Therefore, it is imperative to identify and characterize these pathogens and to evaluate the interactive effects of various pathogens involved in the disease complex. Keeping in view the significance of the problem, associated microflora from wilted plant and its rhizosphere, responsible for SDS of chili was isolated and characterized. Moreover, the individual and combine effect of wilt causing pathogens was also ascertained in the development of SDS in chilies.

MATERIALS AND METHODS

Isolation of Microflora: Chilli plants exhibiting the typical symptoms of green wilting, browning of vascular tissue, rotted roots and infected seeds were collected from Rawalpindi and Islamabad. Roots, vascular tissues, seeds and soil sample (rhizosphere soil of infected plants) were used for the isolation of the pathogens as described by Jamiolkowska (2007), Ishfaq and Khan (2011), Chaudary and Rashid (2011) and Poucke *et al*. (2012). Potato dextrose agar (PDA), Nutrient agar (NA), Corn meal agar (CMA) and Triphenyl tetrazolium chloride (TTC) media were used for the isolation of fungus, bacteria, *P. capsici* and *R. solanacearum* respectively.

Identification of Fungal Pathogens: Morphological characteristics of cultures were studied by microscopic observation under compound microscope at different magnifications and growth characteristics like colony color, texture, growth, pigmentations, hyphae / mycelial color and sclerotium color were observed in Petri plates. *Fusarium* species were identified according to Nelson *et al.* (1981) and *Rhizoctonia* species were identified using the method of Krownland and Staglini (1988).

Identification of Bacterial Pathogens: *Ralstonia solanacearum* were identified as based on their morphological, cultural and biochemical tests. For morphological and cultural characteristics isolates of *R. solanacearum* were streaked on TTC medium and observed for the appearance of colonies after 48 h of incubation. Colonies were observed for odor, size, shape and fluidity. Different biochemical tests such as gram staining (Schaad 1980), KOH (Suslow *et al*. 1982), florescence (King *et al*. 1954) Kovacs oxidase, Catalase test (Schaad 1980), Levan production from sucrose (Schaad 1980), lipase activity on Tween 80 (Sierra 1957) and fermentation or oxidation of glucose (Hayward 1964) were performed for identification, characterization and genus confirmation of *R. solanacearum*.

Role of Pathogens in SDS of Chilli: In order to determine the role of pathogens in sudden death syndrome of chilies, the pathogens were first subjected individually to the pathogenicity tests on susceptible chilli cultivar, California wonder under greenhouse conditions in sterilized soil using the methodology of Naz *et al*. (2008). After confirmation of their pathogenicity the disease complex study was evaluated through different combinations of pathogens to determine the role of individual pathogen in SDS and its role in combination with other pathogens. Pathogenic behavior of only wilt causing pathogens i.e. *F. oxysporum*, *R. solani, R. solanacearum*, *V. dahliae* and *P. capsici* were studied and inoculum's preparation and inoculation methods were followed by Babay-Ahri *et al.* (2009) and Yu *et al.* (2011). Sequential and simultaneous inoculation methods were used to study the interrelationships among pathogens. In case of simultaneous inoculation the chili seedlings were inoculated simultaneously with mixed cultures of pathogens. In case of sequential inoculations, the seedlings were first inoculated with one pathogen after 3-4 days prior to being inoculated with other pathogen. Treatments were replicated thrice. The experiment was also repeated for the confirmation of the results.

RESULTS AND DISCUSSION

On the basis of morphological and cultural characteristics, *F. oxysporum*, *R. solani, R. solanacearum, Rhizopus, Mucor, Alternaria, Penicillium* and *Aspergillus* spp. were identified. Isolation from the diseased vascular tissues and infected seeds showed the presence of *R. solanacearum*. Roots were found infested with *F. oxysporum* and the isolation from soil showed the presence of *Rhizopus, F. oxysporum*, *R. solani, Mucor, Alternaria, Penicillium* and *Aspergillus* species. *F. oxysporum* was isolated on PDA medium and could not be grown on other media*. R. solani*, *Rhizopus, Mucor* and *Aspergillus* spp. demonstrated positive growth on PDA and MEA media. These, however, exhibited no growth on CMA media. *Alternaria* and *Penicillium* spp. exhibited positive growth on PDA, MEA and CMA media. *Ralstonia solanacearum* on the other hand showed positive growth on TTC medium and was unable to grow on NA medium.Among all bacterial and fungal isolates, only *Ralstonia solanacearum, F. oxysporum* and *R. solani* were found to be pathogenic causing wilting, root rot and damping off when inoculated on chilli seedlings. Pathogenicity of these pathogens has been proved by many researchers (Ares *et al*., 2005; Lopez *et al*., 2009; Baby-Ahari *et al*., 2009).

Identification and Characterization of *Fusarium oxysporum* and *Rhizoctonia solani*: The colony appearance of *F. oxysporum* isolates was cottony, thin flat to fluffy, thread like spreading at periphery and looking wet. Growth of the colony was delicate and wooly to cottony becoming felted and wrinkles in mature cultures. The color of the colonies varied from creamy white to white while some isolates produced pinkish violet pigments in colonies after 15 to 20 days of incubation at 25^{o}C on PDA medium (Fig. 1a). These results were similar as reported by Gupta *et al*. (2010). The morphological studies revealed that *F. oxysporum* produced three types of spores .i.e. microconidia, macroconidia and chlamydospores. Microconidia were abundant, one septate, oval to ellipsoidal in shape hyaline and borne on short and plump monophialides and measured 3.31 -10.21 × 2.01 -3.51µ. Macroconidia were produced in sporodochium, thin walled, delicate, 3 to 5 septate, cylindrical, straight to curved, sickle shaped pointed at both ends and measured 22.69-30.76×3.42-4.24 µ (Fig. 1b). Chlamydospores were formed in old culture, which were spherical, 1 celled, smooth and thick walled, terminal or intercalary produced singly or in pairs and measured 9.42-16.15x8.01-12.01µ (Fig. 1c).The results are in line with the findings of Leslie & Summerell (2006) Nelson *et al*. (1981) and Alexopoulus *et al*. (1996).

Rhizoctonia solani produced dark brown, shiny golden and thread like hyphae on PDA medium after two days of incubation. Hyphal branching tends to appear at right angle with constriction and a septum near each branch. Small and oval shape moniloid cells were produced in clusters and the aggregates of these cells are called sclerotia which generally produced in the middle of the colony after 7-10 days of incubation (Fig. 2 a, c). In all isolates the width of the hyphae varied between 3 to 4 µm (Fig. 2 b). The results are in accordance with the findings of Sharma *et al.* (2005), Guleria *et al*. (2007), Thind and Aggarwal (2008) and Goswami *et al.* (2010).

Identification and characterization of *R. solanacearum*: *R. solanacearum* colonies on TTC medium were appeared after 48 h of incubation at 28^{0}C. Variation in colonies was observed. Virulent colonies of *R. solanacearum* were appeared as small, mucoid, opaque irregular, whitish with reddish pink color in the center and brown pigmentation or brown staining (typical for *R. solanacearum*) of medium around the colonies (Fig. 3a) while a virulent colonies appeared uniformly around, smaller and darker in color then virulent. It is also essential to note that *R. solanacearum* is a slow growing bacterium and colonies that were appeared less than 48 h and 28^{0}C were not *R. solanacearum.* All the isolates were quiet similar in their morphological and physiological characters. Similar results were found when the characteristics of isolated bacteria compared with the characters of *R. solanacearum* as described in literature. It may be suspected that the isolated bacterium was *R. solanacearum*. The individual colonies that showed typical *R. solanacearum* characters were selected and then multiplied on 523 medium (Fig. 3b) for further confirmatory tests. These results are in agreement with Kelman (1954), Kado and Heskett (1990), French *et al*. (1995), Garcia *et al*. (1999) and Nouri *et al*. (2009).

Biochemical tests for confirmation of *R. solanacearum*: Biochemical test indicated that the isolates which gave positive results in the following tests: Loop formation, Oxidase test, Catalase test, Production of white dense precipitate around bacterial colonies on Tween 80 and oxidation of glucose whereas they gave negative results in Gram

reaction, Levan production from sucrose and behaved non fluorescent under UV light. According to our results, only 52 % isolates were found similar to each other while remaining 48% differed among themselves. The results of morphological, cultural and biochemical tests of strains were compared with the properties of *Ralstonia* spp. as described in literature. The strains were related to *Ralstonia* spp. and exhibiting high similarities. We identified this isolated strain as *R. solanacearum* belong to the genus *Ralstonia*. The results are in accordance with the findings of Lemessa and Zeller (2007) and Ramesh and Phadke (2012).

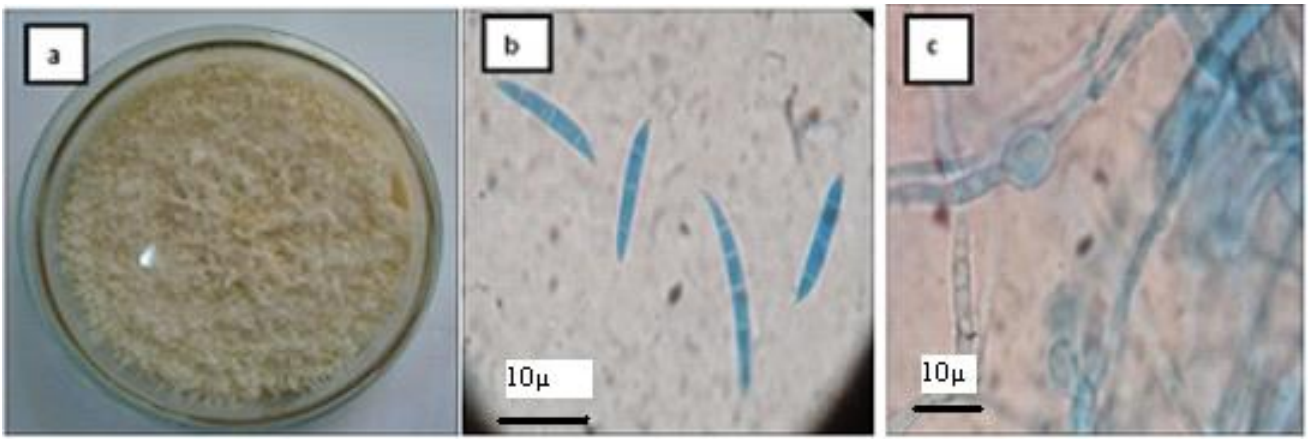

Figure 1. Creamy white growth of *F. oxysporum* on MEA medium (a), Macroconidia of *F. oxysporum* (b) and Chlamydospores of *F. oxysporum*(c).

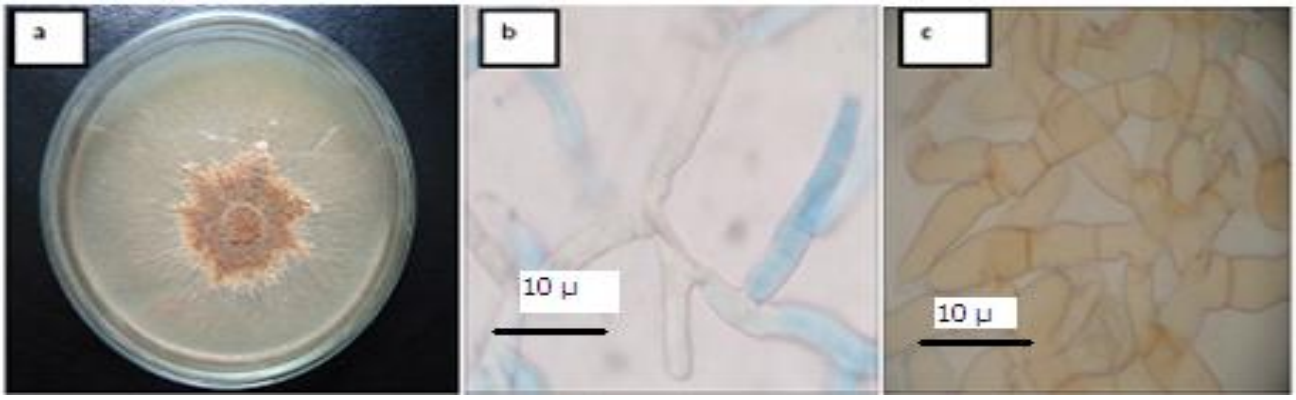

Figure. 2. A = Mycelial and sclerotial growth of *Rhizoctonia solani,* b= Hyphae of *R. solani*, c=Sclerotia of *R. solani* (100X).

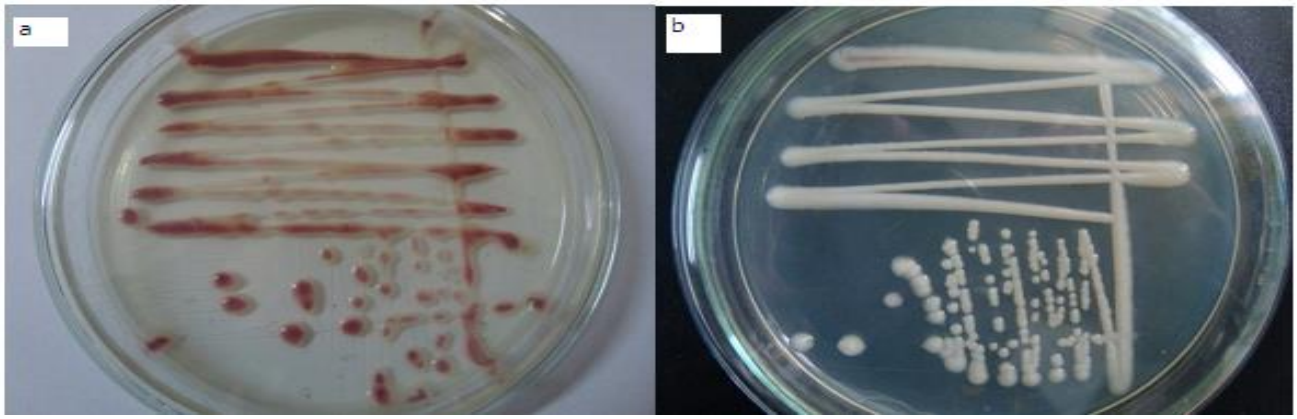

Figure 3. a= Pinkish red centered colonies of *Ralstonia solanacearum* on TTC medium b= Colonies of *Ralstonia solanacearum* on 523 media.

Pathogenicity of *R. solanacearum*: Damping off disease caused by *Rhizoctonia* species produced pre-emergence and post-emergence damping off on seedlings inoculated experimentally. The pathogenic abilities of isolates Rh-TX 9, Rh-CS 1, Rh-AD 4, Rh-TX 6 and Rh-AD 10 were tested on California wonder under greenhouse conditions. Pre-emergence damping off was recorded 15 days after planting, while post-emergence and survived plants were counted up to 40 to 45 after planting (Fig. 4c). The percentages of pre-emergence, post emergence and survival plants were calculated by using the formulae described by Moataza (2006). All isolates were variable in their pathogenic potential. Our results demonstrated that most of the aggressive isolates exhibited pre-emergence damping off. Isolate Rh-TX 9 and Rh-CS 1 were highly pathogenic. The isolates Rh-AD 4 and Rh-TX 6 exhibited moderately susceptible response to California wonder and the isolate Rh-AD 10 proved least virulent it produced only the pre-emergence damping off and resulted in the maximum number of surviving plants. All the seedlings grown in *R. solani* infected soil showed the symptoms of water soaking and dark brown sunken lesions on the lower stem near the soil line. As the infection progressed, the severely infected plants produced wire stem and fell over.

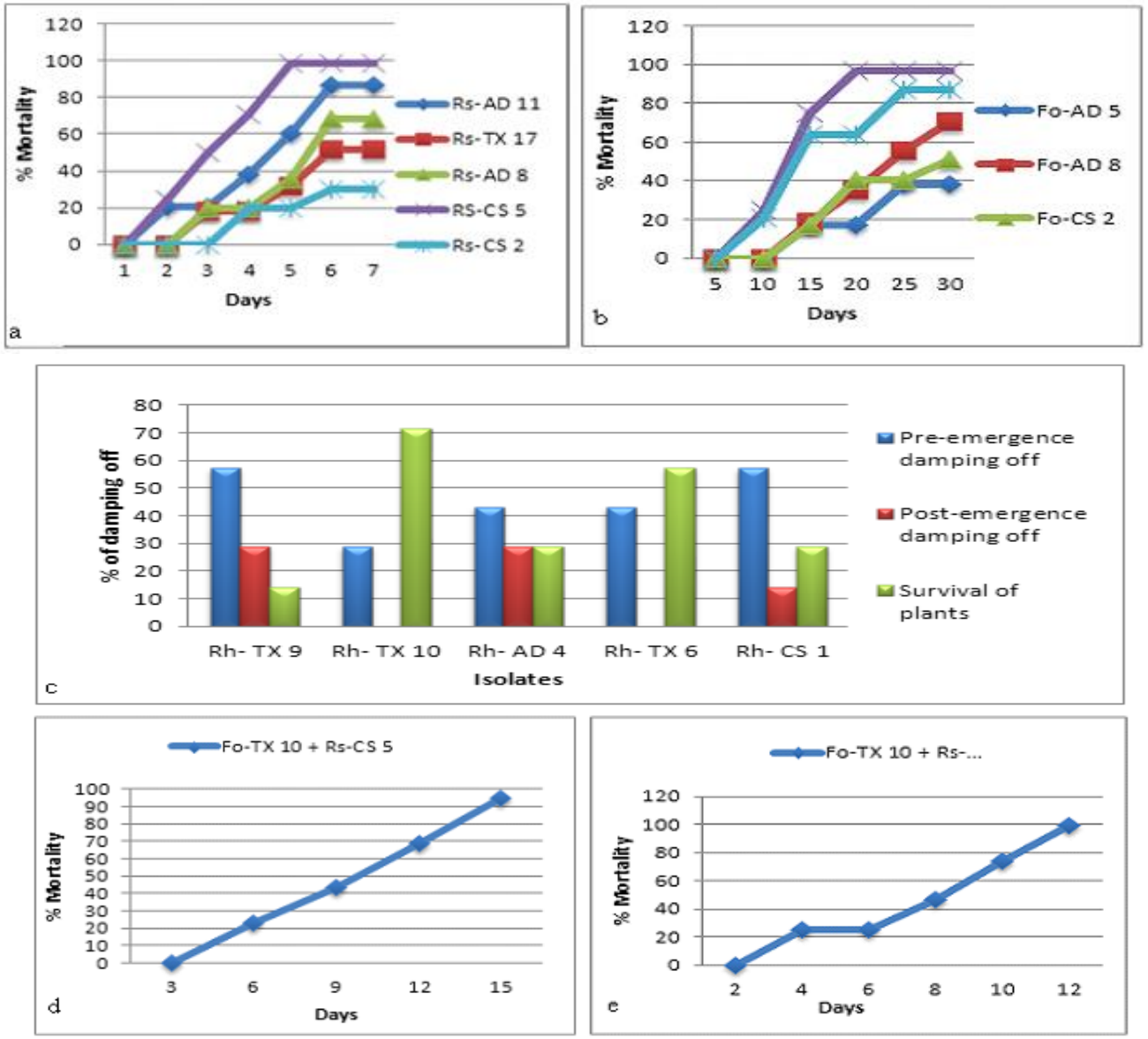

Figure 4. Mean % wilt program in *R. solanacearum* (a) *F. oxysporum* isolates (b) *R. solani* isolates (c) combine effect of *F. oxysporum* and *R. solanacearum* (d) combine effect of *F. oxysporum*, *R. solanacearum* and *R. solani.*

No symptoms were observed on control plant throughout the experimentation period. The results are in the coincidence with the findings of Bolkan and Ribeiro (1985).

Interactive Effect of *R. solanacearum* and *F. oxysporum:* Interactive effect of *R. solanacearum* and *F. oxysporum* was evaluated through sequential inoculation in which the seedlings (CaliforniaWonder) inoculated with most aggressive isolate Rs-CS 5 of *R. solanacearum* prior to inoculate with virulent isolate Fo-TX 10 of *F. oxysporum* after 3 days. In the present study it was inferred that the prior inoculation of *F. oxysporum* has no impact on *R. solanacearum* infection and no significant difference was observed between the disease progress trend in the interactive effect of *R. solanacearum* with *F. oxysporum* and in both pathogens individually (Fig. 4d). Similar results were found by Lin (2001). However, the synergism of *Meloidogyne incognita* with *F. oxysporum* and *R. solanacearum* has been found to augment the seedling mortality, severe wilting and root rot (Shyla 1998; Vijayakumari 2004; Ramaprasad 2005).

Interactive Effect of *R. solanacearum* and *R. solani*: The combined effect of *R. solanacearum* and *R. solani* was evaluated through simultaneous inoculation under greenhouse condition. Results showed that no symptoms were observed and all the inoculated plants remained healthy even after thirty days of inoculation. The results obtained in this study suggest that this combination of pathogens have the disease suppressive effect where one pathogen suppresses the pathogenic behavior or virulence of the other pathogen.

Interactive Effect of Wilt Causing Pathogens: Disease complex involving *R. solanacearum*, *F. oxysporum* and *R. solani* was evaluated through sequential inoculation in green house conditions. The highly virulent strains Rs-CS 5, Fo-TX 10 and Rh-TX 9 of these pathogens were selected to study their interaction. Results showed that necrosis of stem and wilting of leaf were more apparent within 3 to 4 days in seedlings inoculated sequentially than in plant inoculated with these pathogens alone. Most of the plants were completely collapsed within 7 to 12 days of post inoculation with very rapid disease progression (Fig. 4e). It appeared that this combined infection resulted in rapid wilting and sudden death of the seedlings. The interactive relationship among these pathogens leads to the disease complex as a synergistic or additive relationship. Such symptoms were not observed in plants inoculated with these pathogens alone. Control plants exhibited no symptoms. The severity in SDS might be due to the two fungal wilt causing pathogens *F. oxysporum* and *R. solani* both inducing wilt complex and the presence of both these pathogens resulted in earlier expression of wilt symptoms and the effect of both pathogens were more pronounced and have a significant impact on chili seedlings as compared to the other combinations of pathogens (Moens and Ben-Aicha 1990; Ehteshamul-Haque and Ghaffar, 1994). Moreover, the major problem of chili plantation are mostly caused by interactive effect of *F. oxysporum* and *R. solani,* and their synergistic effect results in rapid mortality of the seedlings followed by rotting of roots and induces SDS in chili (Abdel-Kader *et al*., 2004).

CONCLUSIONS

Morphological, cultural and biochemical characteristics confirmed the presence of *F. oxysporum*, *R. solani* and *R. solanacearum* and pathogenicity tests also confirmed these pathogens are the causal agents of sudden death syndrome of chillies. It was further confirmed through complex studies in which staggering increase in wilting percentage was observed when the seedlings inoculated with three pathogens sequentially.

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