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Exploiting cucurbitaceous species as rootstocks for management of *Fusarium* wilt (*Fusarium oxysporum*) in bitter gourd

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Abstract

Bitter gourd is affected by various kinds of pathogens, of which *Fusarium* wilt *Fusarium oxysporum* plays a vital role. The present investigation focuses on the role of defense related enzymes which impart resistance against *Fusarium* wilt. A total of ten cucurbitaceous rootstocks and two bitter gourd scions were screened against *Fusarium* wilt pathogen under *in vitro*. Results on screening against *Fusarium* wilt revealed that *Citrullus colocynthis*, *Cucumis metuliferus* and *Cucurbita moschata* exhibited no symptom and manifested as resistant to *Fusarium* wilt and the least percent incidence of 21.62, 37.44 and 48.90 was observed in *Luffa cylindrica* followed by *Momordica charantia* var. *muricata* rootstock (23.58, 42.18 and 50.34) at 30, 45 and 60 days after inoculation. Seedlings of aforementioned species were harvested at 0, 7, 14, 21, 28 and 35 days after challenge inoculation and assayed for defense related enzymes activity. Significant increases in the activities of peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) activity was observed in resistant rootstocks *viz.*, *C. colocynthis*, *C. metuliferus* and *C.moschata followed* by moderately resistant rootstocks *viz.*, *M. charantia* var. *muricata* and *L. cylindrica*. Native page analysis of both PO and PPO isozymes was carried out for the time course and examined. Isozyme analysis revealed that unique PO and PPO isozymes were induced in aforementioned resistant rootstocks. From this study point out that the cucurbitaceous species *viz.*, kumatikai *(C. colocynthis)*, African horned cucumber *(C. metuliferus)* and pumpkin (*C. moschata)* with high or moderate levels of these biochemical constituents suffered less for *Fusarium* wilt pathogen and these rootstocks served as the best rootstocks for grafting with bitter gourd scions followed by mithipakal(*M. charantia* var. *muricata)* and sponge gourd *(L. cylindrica)*.

Keywords: *Fusarium* wilt, grafting, cucurbitaceous rootstocks, bitter gourd.

Introduction

Bitter gourd (*Momordica charantia* L.) is one of the important cucurbitaceous vegetable grown in India. Among the cucurbits, it is considered a prized vegetable because of its high nutritive value especially ascorbic acid and iron (Behera, 2004). Depending on location, bitter gourd is also known as bitter melon, Karela or Balsam pear. The immature fruits and tender vine tips are used in a variety of culinary preparations. It is a most common vegetable cultivated throughout India during warm season (Satkar et al., 2013). Bitter gourd has been used in various herbal medicine systems for a long time because of its disease preventing and health promoting phytochemical compounds like dietary fiber, minerals, vitamins, flavonoids and antioxidants. It is also used for reduction of blood sugar levels in the treatment of type-2 diabete (Singh et al., 2013).

The crop is cultivated over an area of 80990 ha in India with an annual production of 8, 30, 450 tonnes and the productivity of 10.25 tha^{-1} (www.indiastat.com, 2015-16). The main problem with bitter gourd production in India is *Fusarium* wilt and root knot nematode. (Tamilselvi, 2014). *Fusarium* wilt is caused by *Fusarium oxysporum* is responsible for a wider range of diseases on economically important crops including bitter gourd, watermelon and cucumber. It is the most devastating soil borne disease and one of the major yield limiting constraint which cause profound economic losses ranging from 30 to 50 per cent under dry warm conditions (Tamilselvi and Pugalendhi, 2015). *Fusarium* wilt symptoms include damping-off, seedling disease or wilt during any stage of plant development. Symptoms on mature plants typically appear following fruit set and may appear as a dull grey green appearance of the leaves followed by yellowing of the crown foliage, wilting during the day and eventual death. Brown stripes will develop on stems and branches of infected plants. Vascular discoloration is visible inside stem and stem collars often turn dark brown. There are different ways to circumvent these soil diseases such as culturally by crop rotation, biological control, soil fumigation by the use of chemicals and development of resistant varieties through breeding programs. The management practices have some disadvantages. Hence, the selection of resistant rootstocks to soil borne pathogens seems to be an effective solution

Aside from the initial reports, little information on the pathogenicity of *F. oxysporum* is available. This knowledge would be important for identification of resistant rootstocks against soil borne diseases. This is particularly important as

cucurbitaceous species were often used as rootstocks for grafting in bitter gourd, cucumber and watermelon to control *Fusarium* wilt and other soil borne diseases. In view of this fact, the cucurbitaceous rootstocks and bitter gourd scions were screened against *Fusarium* wilt pathogen under pot culture to identify the resistant species for further studies.

The defense enzymes viz., Peroxidase (PO), Polyphenol oxidase (PPO) usually accumulates upon wounding in plants which catalyze the formation of lignin and other oxidative phenols. Phenylalanine ammonia lyase is the key enzyme in inducing synthesis of salicylic acid (SA), phytoalexins and phenolics which induces systemic resistance in many plants. However, screening of cucurbitaceous rootstocks against *Fusarium* wilt and observation on involvement of defense related enzymes were limited. Hence, the present study aimed to evaluate the involvement of defense enzymes such as peroxidase(PO),Polyphenol oxidase (PPO) and Phenylalanine ammonia lyase (PAL) in cucurbitaceous rootstocks and bitter gourd scions after challenge inoculation with *Fusarium oxysporum.*

Results

Response of cucurbitaceous rootstocks and bitter gourd scions against Fusarium wilt incidence

A pot culture experiment was conducted to study the response of cucurbitaceous rootstocks and bitter gourd scions to *Fusarium* wilt. Findings of this study indicated that, kumatikai (*C. colocynthis*), African horned cucumber *(C. metuliferus)* and pumpkin (*C. moschata*) exhibited no symptom and manifested as resistant to *Fusarium* wilt and sponge gourd (*L. cylindrica*) and mithipakal (*M. charantia* var. *muricata*) showed moderately resistant reaction under *in vitro* (Table 1).

Biochemical basis of defence response in cucurbitaceous rootstocks and bitter gourd scions

In addition, biochemical defense mechanism against *Fusarium* wilt in cucurbitaceous rootstocks and bitter gourd scions were studied. The PO activity was assayed in aforementioned species from 0, 7, 14, 21, 28 and 35 days after challenge inoculation. The PO activity was induced after inoculation and increased activity was observed from seventh day to 21st day after inoculation and thereafter gradual decrease in activity was recorded. The highest activity of PO was observed in kumatikai *(C. colocynthis)* (3.82 changes in OD $min^{-1}g^{-1}$ of root) followed by African horned cucumber *(C. metuliferus)* (3.25 changes in OD $min^{-1}g^{-1}$ of root) and pumpkin *(C. moschata) (*2.90 changes in OD $min^{-1}g^{-1}$ of root) (Fig 1). The two bitter gourd scions (Palee F_1 and CO 1) showed a slow increase in PO activity from seven days after inoculation and showed a declining trend at 35 days after inoculation.

PPO activity was also measured in the roots of *Fusarium* wilt pathogen inoculated cucurbitaceous rootstocks and bitter gourd scions. The plants showed increased level of polyphenol oxidase activity and reached the highest level on 21st day after inoculation thereafter declined. The highest PPO activity was recorded in kumatikai *(C. colocynthis)* (3.23 changes in OD $min^{-1}g^{-1}$ of root) followed by African horned cucumber *(C. metuliferus)* (3.14 changes in OD $min^{-1}g^{-1}$ of root) and pumpkin *(C. moschata)* (3.08 changes in OD $min^{-1}g^{-1}$ of root) (Fig 2).

The cucurbitaceous rootstocks and bitter gourd scions were also analyzed for phenylalanine ammonia lyase (PAL) activity after inoculation with *Fusarium* wilt pathogen. The results revealed that the plants induced to synthesize higher level of PAL enzyme activity. The activity of enzyme reached the highest level on 21 days after challenge inoculation and thereafter declined with decreasing rate. The highest PAL activity among the rootstocks was noticed in kumatikai (*C. colocynthis*) (31.59 nmol of trans cinnmic acid min^{-1} g^{-1} of root) followed by African horned cucumber (*C. metuliferus*) (31.20 nmol of trans cinnmic acid min^{-1} g^{-1} of root) and pumpkin (*C. moschata*) (29.71 nmol of trans cinnmic acid min^{-1} g^{-1} of root) (Fig 3).

Native gel electrophoresis was done to study the isozyme variation among the cucurbitaceous rootstocks and bitter gourd scions. The roots of the aforementioned species were taken at 21 days after inoculation.

The results of the PO isozyme analysis revealed that, number and intensity of isoforms was varying among the species.

More number of isoforms (PO1, PO2, PO3 and PO 4) was observed in the resistant rootstocks *viz.*, Kumatikai (*C. colocynthis*), African horned cucumber (*C. metuliferus*) and pumpkin (C. *moschata*) followed by moderately resistant rootstocks viz., Mithipakal (*M. charantia* var. muricata) and sponge gourd (*L. cylindrica*) compared to susceptible rootstocks and bitter gourd scions (Supplementary Figure 1).

Native gel electrophoretic separation of enzyme extract from the cucurbitaceous rootstocks and bitter gourd scions showed PPO isoforms. Among the cucurbitaceous rootstocks and bitter gourd scions, the rootstocks *viz.*, Kumatikai (*C. colocynthis*), African horned cucumber (*C. metuliferus*) and pumpkin (*C. moschata*) found to be resistant. At 21 days after challenge inoculation with *Fusarium* wilt pathogen showed more number of isoforms but the other species showed only less isoforms with less intensity (Supplementary Figure 2).

Discussion

Findings of this study indicated that, kumatikai *(C. colocynthis),* African horned cucumber *(C. metuliferus)* and pumpkin *(C. moschata)* exhibited no symptom and manifested as resistant to *Fusarium* wilt followed by sponge gourd *(L. cylindrica)* and mithipakal (*M. charantia* var. *muricata) rootstocks* showed moderately resistant reaction under *in vitro.* There was no earlier report in India regarding resistance to *Fusarium* wilt and this would be the first record of evidence to show that these species are resistant to *Fusarium* wilt. But the available reports from foreign countries showed that muskmelon grafted onto *C.metuliferus*, *C.maxima* and *C.moschata* showed resistance against *Fusarium* wilt (Nisini et al., 2002). Mohamed et al. (2012) reported that grafting watermelon onto inter-specific hybrid 'Nun 6001' (*Cucurbita maxima* x *Cucurbita moschata)* also showed resistant reaction to this disease.

On infection with *Fusarium* wilt pathogen, the activity of the enzyme increased significantly in resistant species, which in turn led to formation of more quinones and other oxidation products, resulting in reduced multiplication and inactivation of the pathogen (Singh and Singh, 1989). Higher level of expression of defense-related proteins and timely accumulation of chemicals at the infection site certainly prevent the colonization of pathogen in the plant species (Saravanakumar et al., 2007).

Among the different defense gene products, peroxidases are important in conferring resistance against many pathogens. PO represents important component of an early response in plant pathogen attack and play a key role in biosynthesis of lignin which limit the extent of pathogen spread (Bruce and

Table 1. *Fusarium oxysporum* incidence on cucurbitaceous rootstocks and bitter gourd scions under *in vitro*.

Rootstocks	*Fusarium* wilt incidence (%)		
	30* DAP	45* DAP	60* DAP
Mithipakal (*Momordica charantia* var. *muricata)*	23.58 (4.90) ± 1.8	42.18 (6.83) ±3.2	50.34 (7.40) ±3.7
Fig leaf gourd *(Cucurbita ficifolia)*	48.21 (6.97) ±3.7	62.63 (7.94) ±4.3	78.35 (8.87) ±5.2
Pumpkin *(Cucurbita moschata)*	0 (0.70) ± 0.0	0 (0.70) ±0.0	0 (0.70) ±0.0
Zucchini squash *(Cucurbita pepo)*	63.32 (7.98) ±4.5	78.94 (8.91) ±5.4	92.30 (9.63) ±6.6
Sponge gourd *(Luffa cylindrica)*	21.62 (4.70) ±1.5	37.44 (6.15) ±2.7	48.90 (7.30) ±3.5
Ridge gourd *(Luffa acutangula)*	42.44 (6.55) ±2.9	65.37 (8.11) ±4.5	80.71 (9.01) ±5.5.
Bottle gourd *(Lagenaria siceraria)*	46.84 (6.88) ±3.2	74.67 (8.67) ±5.3	88.41 (9.42) ±6.3
Ash gourd *(Benincasa hispida)*	48.33 (6.98) ±3.8	53.62 (7.35) ±3.4	84.94 (9.24) ±6.2
Kumatikai *(Citrullus colocynthis)*	0 (0.70) ±0.0	0 (0.70) ±0.0	0(0.70) ±0.0
African horned cucumber *(Cucumis metuliferus)*	0 (0.70) ±0.0	0 (0.70) ±0.0	0 (0.70) ±0.0
Scions			
Momordica charantia cv. Palee F_1	46.22 (6.83) ±3.5	64.66 (8.07) ±5.7	80.94 (9.02) ±6.4
Momordica charantia cv. CO 1	50.55 (7.14) ±4.0	78.56 (8.89) ±5.5	89.38 (9.48) ±6.3
SEd	0.5318	0.6463	0.7088
CD at (0.05)	1.1029	1.3403	1.4699

Figures in parentheses are square root (X+0.5) transformed values.

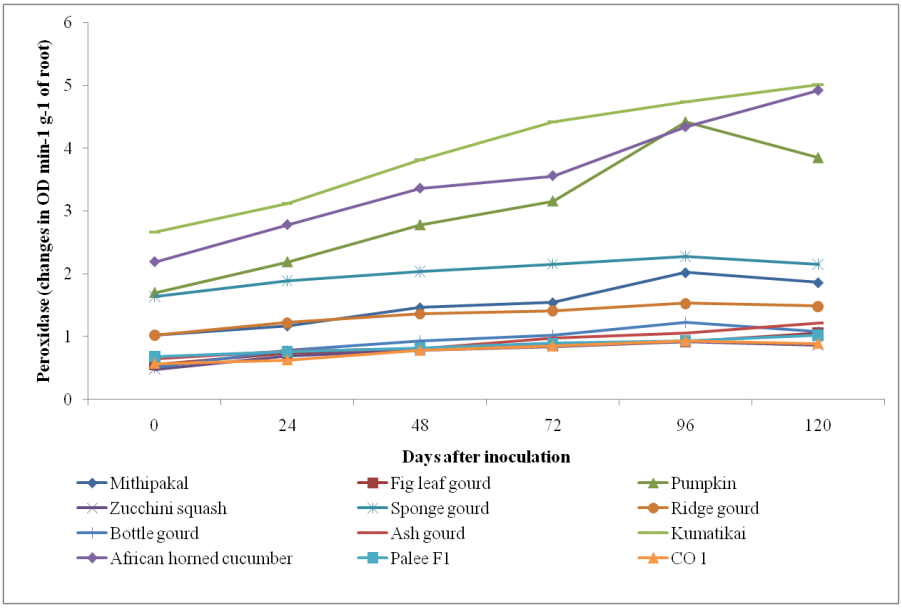

Fig 1. Induction of PO activity in cucurbitaceous rootstocks and bitter gourd scions against *Fusarium oxysporum*.

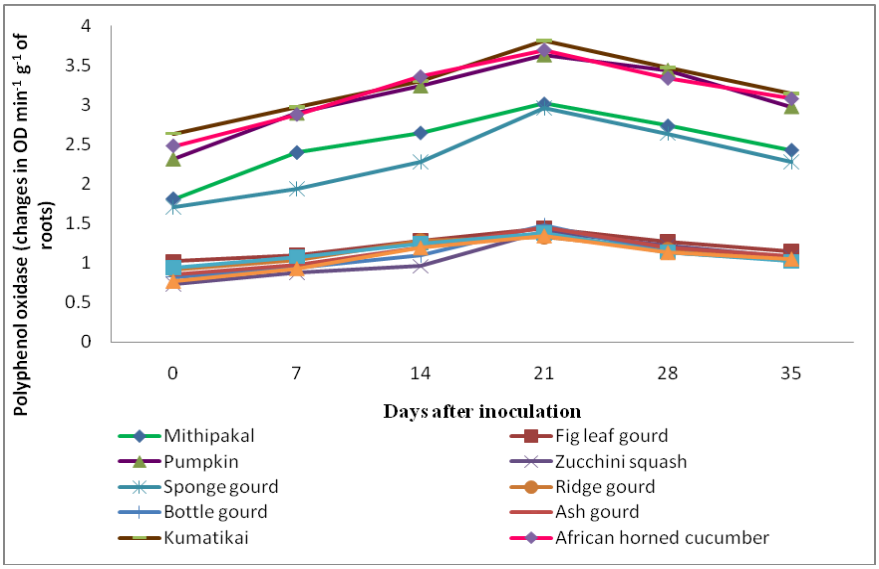

Fig 2. Induction of PPO activity in cucurbitaceous rootstocks and bitter gourd scions against *Fusarium oxysporum*.

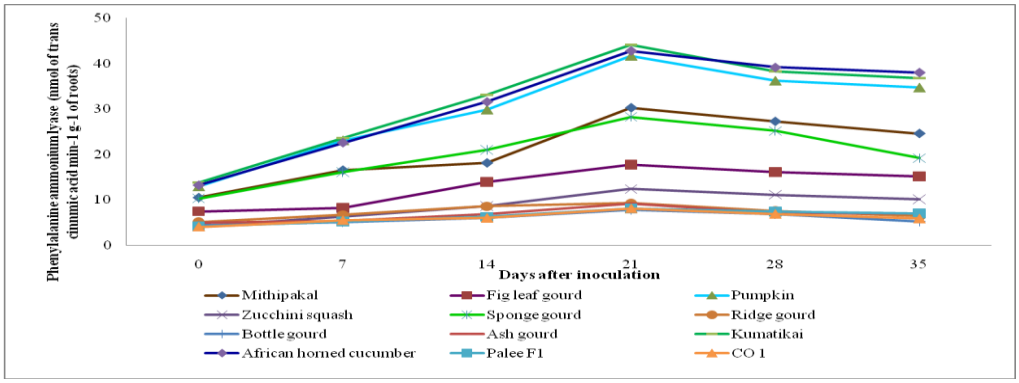

Fig 3. Induction of PAL activity in cucurbitaceous rootstocks and bitter gourd scions against *Fusarium oxysporum.*

West, 1989). The product of this enzyme is the presence of a hydrogen donor and hydrogen peroxidase have antimicrobial activity and even antiviral activity (Van and Callow, 1983). In this study, increased PO activity was observed in kumatikai *(C. colocynthis),* African horned cucumber (*C. metuliferus)* and pumpkin *(C. moschata)* at different intervals after inoculation. The enzyme level was increased after pathogen reaction and reached the peak at 21 DAI, then gradually decreased. Similar such observation of increased PO activity was also reported by Kohatsu et al. (2013) when cucumber plants grafted onto 'Shelper' squash and 'Green-stripped cushaw squash against soil borne diseases.

PPO is induced via octadecanoid defense signal pathway which is usually associated with feeding by insects or similar physical trauma. It involves jasmonic acid as an intermediate signal and culminates in the production of proteins such as PPO and proteinase inhibitors (Schaller and Ryan, 1995). The PPO activity varied significantly among rootstocks and bitter gourd scions under *in intro*. Susceptible rootstocks and bitter gourd scions had lower polyphenol oxidase activity. Significantly elevated PPO activity was observed in resistant rootstocks *viz.,* kumatikai (*C. colocynthis*), African horned cucumber (*C. metuliferus*) and pumpkin (*C. moschata*) and it catalyses the last step of biosynthesis of lignin and other oxidative phenols. As sown in Figure 2, the PPO activity increased significantly 7 days after challenge inoculation and reached the highest level at 21 days after inoculation then gradually decreased. These observations are in line with the findings of Sherly (2010) in brinjal grafted onto *S. torvum* rootstock challenged with dry root rot pathogen and cucumber plants grafted onto 'Shelper' squash and 'Green-stripped cushaw' squash against soil borne diseases (Kohatsu et al., 2013).

PAL is the key enzyme in inducing the synthesis of salicylic acid (SA), which is responsible for inducing systemic resistance in many plants. The time required to activate the defence mechanisms is important for suppression of the invading pathogen. The increased activity of PAL constituted for enhancing the resistance in kumatikai *(C. colocynthis),* African horned cucumber *(C. metuliferus)* and pumpkin *(C. moschata)* against fungal pathogen and these rootstocks excelled for this trait. Earlier and higher level of expression of PAL activity was observed in resistant rootstocks throughout the experimental period. The enzyme activity was remain constant in susceptible rootstocks and bitter gourd scions. Several studies have shown that induction of PAL activity in *Colletotrichum capsici* resistant chilli genotypes (Jabeen et al., 2009) and in *Solanum torvum* rootstock against dry root rot pathogen (Sherly, 2010). These results indicated that all these biochemical constituents are responsible for conferring resistance to *Fusarium* wilt pathogen. These findings are in line with Sherly (2010) in brinjal for *M. incognita* and dry root rot pathogen (*Macrophomina phaseolina*).

Findings of this study point out that the rootstocks *viz.,* kumatikai (*C. colocynthis*), African horned cucumber (*C. metuliferus*) and pumpkin (*C. moschata*) with high or moderate levels of these biochemical constituents suffered less with *Fusarium* wilt disease and these rootstocks served as the best rootstocks for grafting with bitter gourd scions followed by mithipakal (*M. charantia* var. *muricata)* and sponge gourd *(L. cylindrica)*.

Isozyme analysis by electrophoresis provides a well defined and effective method to detect genetic differences among individuals. Among the organic molecules, isozymes are very useful, which aid in comparing the genotypes, though they are used only as a supplementary tool along with morphological, genetical or other biochemical methods. In the present investigation, isozyme pattern of PO and PPO were studied in cucurbitaceous rootstocks and bitter gourd scions at 21 days after challenge inoculation with *Fusarium* wilt pathogen. In PO and PPO isozyme analysis more isoforms were present in resistant rootstocks *viz.,* kumatikai *(C. colocynthis),* African horned cucumber *(C. metuliferus)* and pumpkin *(C. moschata).* However moderately resistant species *viz.,* Mithipakal (*M. charantia* var. *muricata)* and sponge gourd *(L. cylindrica)* also have isoforms which are lesser than resistant species but higher than isoforms which present in susceptible rootstocks and bitter gourd scions.

With respect to PPO, the susceptible rootstocks and bitter gourd scions had faint bands. In resistant species there was an increase in isoform expression with improved expression of band followed by moderately resistant species. The results of the present study on isozyme profile revealed that there was an improved banding pattern with respect to the intensity of the band and also with the increase in the band number in resistant species followed by in moderately resistant species. This may be attributed to the expression of structural genes, which have expressed after challenge inoculation. This finding falls in line with Sherly (2010) in brinjal against dry root rot pathogen and Ramyabharathi (2011) and Prabhukarthikeyan (2012) against *Fusarium* wilt respectively.

Materials and Methods

Experimental site and Plant materials

The pot culture experiment was carried out from 2013 to 2014 at the Department of plant pathology glasshouse, Tamil Nadu Agricultural University, Coimbatore, India (11^0 N

latitude, 77^0 E longitudes and an altitude of 426.26 m above mean sea level) to evaluate the percent disease incidence and defense reaction of cucurbitaceous rootstocks *viz*., Mithipakal (*M.Charantia* var. *muricata*), kumatikai (*Citrullus colocynthis*),African horned cucumber *(Cucumis metuliferous),* fig leaf gourd (*Cucurbita ficifolia*), pumpkin (*Cucurbita moschata*), zucchini squash (*Cucurbita pepo*), bottle gourd (*Lagenaria siceraria*), ash gourd *(Benincasa hispida*), ridge gourd (*Luffa acutangula*) and sponge gourd (*Luffa cylindrica*) and bitter gourd scions (Palee F_1 and CO 1) against *Fusarium* wilt (*Fusarium oxysporum*). The pathogen was isolated from bitter gourd plants showing typical wilt like symptom of *Fusarium* wilt by using potato dextrose agar (PDA) medium. The stock culture was maintained in Potato Dextrose Agar plates and slants, further sub cultured at monthly intervals.

Screening of cucurbitaceous rootstock and bitter gourd scions for resistance to Fusarium wilt

Fusarium oxysporum multiplied on sand - maize medium (Ricker and Ricker, 1936) was incorporated into sterilized uniform pot mixture (Red soil: Sand: FYM in 2:2:1 ratio) filled in earthen pots at the 100 g /pot. 30 days old rootstocks and scion seedlings were planted in the pots which contain artificially inoculated fungal pathogen and under glasshouse conditions. Five replications were maintained for each rootstocks and bitter gourd scions. The measurement of disease incidence was taken at 15, 30 and 45 days of pathogen inoculation. The disease incidence was assessed using the following formula:

$$\text{Percent disease incidence} = \frac{\text{Total number of plants infected}}{\text{Total number of plants observed}} \times 100$$

Assay for defence related enzymes through biochemical analysis

The biochemical constituents *viz.*, PO, PPO and PAL were estimated in cucurbitaceous rootstocks and bitter gourd scions. Recently matured physically active roots of five randomly selected plants after inoculation were taken for biochemical analysis. Root of aforementioned species were collected at 0, 7, 14, 21, 28 and 35 days after challenge inoculation, washed in running tap water and stored in deep freezer (-80º C) until used for biochemical analysis. Samples obtained from different time interval was homogenized in chilled pestle and mortar with 2 mL of ice cold 0.1 M sodium phosphate buffer (pH 7.0, at 4ºC). The homogenate was centrifuged at 16000 rpm at 4^0C for 15 minutes in a refrigerated centrifuge and the supernatant was used as enzyme source.

Peroxidase (PO) activity

Peroxidase activity was assayed by using the method of Srivastava (1987). The enzyme activity was expressed as change in $ODmin^{-1}g^{-1}$of protein (Hammerschmidt et al., 1982).

Poly phenol oxidase (PPO) activity

PPO activity was determined as in Mayer et al. (1965). The PPO activity was expressed as change in OD $minute^{-1}$ g^{-1} of protein.

Phenylalanine ammonia lyase (PAL) Activity

PAL activity was determined as the rate of conversion of L-phenylalanine to transcinnamic acid at 290 nm and enzyme activity was expressed as nmol transcinnamic acid $minute^{-1}$ g^{-1} tissue (Dikerson et al., 1984).

Native PAGE Analysis of PO and PPO isozymes

To study the expression pattern of different PO and PPO isozymes in wild and cultivated cucurbitaceous rootstocks and bitter gourd scions, Native polyacrylamide gel electrophoresis (Native PAGE) was carried out (Sindhu et al., 1984 and Jeyaraman et al., 1987). After staking, the gel was washed with distilled water and photographed.

Statistical analysis

The experiments were conducted with five replications for each rootstocks and bitter gourd scions. The data were analyzed using IIRISTAT version 92-1 programme developed by the Biometric Unit, International Rice Research Institute, the Philippines. Data were subjected to analysis of variance (ANOVA) using completely randomized design following Gomez and Gomez (1976). Data in percent were square root (X+0.5) transformed before analysis and the values mentioned as Mean±SE.

Conclusion

The study revealed that the rootstocks *viz.*, kumatikai *(C. colocynthis),* African horned cucumber *(C. metuliferus)* and pumpkin *(C. moschata)* with high levels of these biochemical constituents suffered less for *Fusarium* wilt pathogen and these rootstocks served as the best for grafting with bitter gourd scions followed by mithipakal (*M. charantia* var. *muricata)* and sponge gourd *(L. cylindrica)*. These results indicated that all these biochemical constituents are responsible for conferring resistance to *Fusarium* wilt pathogen.

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