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Citrus is an important economic crop of North Bengal. Root rot disease caused by *Fusarium oxysporum* is one of the most serious diseases found in mandarin. In the present study spent mushroom compost (SMC) following cultivation of *Agaricus bisporus* was used both in aqueous and powdered forms for the management of Root rot wilt complex of *Citrus reticulata* caused by *F. oxysporum*. The inhibition of fungal growth by spent mushroom substrate in both *in-vitro* and *in-vivo* was done. Spent composts significantly reduced fungal wilt of citrus plant under green house conditions. Disease severity was reduced by application of SMC which was evident with increased activity of defense enzymes (PAL, POX, and β-1,3-glucanase and chitinase) and cellular localization of β-1,3-glucanase and chitinase were confirmed by indirect immunofluorescence using PAbs of chitinase and glucanase and FITC conjugates. Moreover, SMC improved overall growth of citrus plants in terms of plant height, number of flowers per plant, number of shoots per plant, root length, root weight, and dry weight of plant. The ability of SMC to reduce disease severity and enhance plant growth could be attributed to the alteration of physical structure of soil and encouragement of plant friendly microbes by the compost.

Key words: Spent mushroom compost, *Citrus reticulata*, *Fusarium oxysporum*, root rot

INTRODUCTION

The use of spent mushroom substrate (SMS) as well as spent mushroom compost (SMC) in growing agricultural crop has been recognized in recent times as a possible means of enhancing sustainable agriculture (Jonathan *et al.* 2014; Barman *et al.* 2015, Thakur and Singh, 2020). Spent mushroom substrate has been reported to contain nutrients which could be used for the growth of useful plants. These materials are generally non-toxic to the cultivated plant crops; and it could be used as soil amendment for crop systems (Jonathan *et al.* 2014).

In the crop system, in order to provide balanced nitrogen and carbon for the growing plants, SMS further degrades in the soil to humus which is very important to maintain soil structure, good aeration, and water holding capacity and maximize the fruit crop productivity (Adedokun *et al.* 2013). The addition of spent compost to agricultural field has been found to be an effective soil manure and conditioner and has been found to increase the yield of some leafy vegetable crops (Kadiri *et al.* 2010; Barman *et al.* 2015, Roy *et al.,* 2015). The SMS has potential to bio-remediate several agricultural grade fungicides and pesticides (Ahlawat *et al.* 2011). The yield and quality of different crops systems increase upon using the SMS as manure alone or combination with inorganic fertilizer (Ahlawat *et al.* 2011). Spent mushroom substrate is being used for disease suppression of plant. Plant diseases like *Pythium*-damping off, apple scab, cucumber anthracnose caused by fungal pathogen have been found to be suppressed by using water extract of SMS treatment (Parada *et al*. 2012). Kwak *et al*. (2015) used water extract of SMS as an eco-friendly disease control agent. SMC extract was successfully applied as bio-control agents to suppress the Fusarium wilt of tomato by Adedeji *et al.,* (2016). Antagonistic activity of SMC extract against a broad range of plant pathogens have been demonstrated by Isah *et al*., (2014). Up to 55% disease index was reduced by the treatment of spent mushroom compost (SMC) against *Fusarium oxysporum* in tomato plant (Adedeji *et*

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al. (2016). Disease severity caused by *F. oxysporum, Sclerotium rolfsii* and *Rhizoctonia bataticola* was reduced by SMS treatment (Ahlawat *et al.* 2011). Spent mushroom substrate (SMS) having bio-agents, supply nutrition to the soil as well as helps in management of soil-borne plant pathogens (Verma *et al*. 2017).

In the present investigation spent mushroom compost following cultivation of button mushroom (*Agaricus bisporus*) was exploited for developing management strategies of Root rot disease of mandarin plant (*Citrus reticulata*) caused by *Fusarium oxysporum*.

MATERIALS AND METHODS

Preparation of Spent compost extract

Spent mushroom compost (SMC) following cultivation of button mushroom (*Agaricus bisporus*) was obtained from Mushroom production Unit of Immuno Phytopathology Laboratory, Department of Botany, University of North Bengal. The compost was first shade dried and then powdered with the help of electric grinder. To make organic extracts, powder of SMC was soaked in sterile distilled water for 24 h at the rate of 15 g/100 ml. To remove large particles, the organic aqueous extracts were filtered through Whatman no. 1 filter paper.

In vitro suppression of Fusarium oxysporum by SMC extract

Mycelia of *A. bisporus* and extract of spent mushroom compost (SMC) was initially taken up for their *in vitro* evaluation against root rot fungal pathogen (*F. oxysporum*). For each of the antagonistic tests, 5 mm disc from advancing zone of 7 day old culture of the pathogen (*F. oxysporum*) was taken and placed on the centre of PDA medium mixed with cold sterilized SMC extract of *A. bisporus* kept in Petri plate. Similarly, agar disc (5 mm) of both *F. oxysporum* and *A. bisporus* was placed in the same Petri plate on the opposite end, cold sterilized SMC extract was placed on the medium in between two blocks and allowed to grow. PDA medium without SMC extract was used as Control. Steam sterilized SMC extract mixed with PDA medium was also used as check control to evaluate the inhibition capacity of cold sterilized SMC extract. The percent inhibition in the radial growth was calculated by the following formula-

Percent inhibition = C-T/C × 100
Where C= radial growth in control;
T= radial growth in treatment

In vivo application of SMC on citrus plant and disease assessment

The experiment was conducted in potted condition with twenty replicates. Earthen pots were filled with 1.5 kg dry soil which was amended with 500 g fresh spent compost of *Agaricus bisporus*. One seedling (2 year old) of mandarin (*Citrus reticulata*) was transplanted in each pot and observation was carried out for next two months. After that again 500gm of spent compost of *A. bisporus* was added to the rhizosphere of potted plant earlier amended with SMC, further kept for two weeks and finally inoculated with sand maize meal grown inoculum of *F. oxysporum* with the soil of twenty potted plants (TI) as described by Chakraborty and Chakraborty (1989). Untreated (without SMC) plants were separately inoculated (UI) with the pathogen. SMC treated healthy (TH) and untreated healthy (UH) plants were also kept for observation. The effect and severity of the disease was determined as determined by Chakraborty *et al* (2019). Inoculated plants were examined at an interval of 15 days up to 45 days. Each time, the plants were uprooted, washed, and symptoms were noted. Finally, roots were dried at 60^0C for 96 h and weighed. Root rot index was calculated based on the percentage of root area affected, and they were graded into six groups, and a value was assigned to each group viz. 0**-** no symptoms; 1- small roots turn brownish and start rotting; 2**-** leaves start withering, and 20-40% of roots turn brown; 3**-** leaves withered and 50% of roots affected; 4**-** shoot tips also start withering; 60-70% roots affected; 5**-** shoots withered with defoliation of lower withered leaves, 80% roots affected; 6**-** whole plants die, with upper withered leaves remaining attached; roots fully rotted. The root rot index in each case was the quotient of the total values of the replicate roots and the number of roots (i.e., number of plants).

Extraction and assay of defense enzymes **ß-*1,3- glucanase* (*E.C. 3.2.3.39*)**

Extraction of ß-1,3-glucanase was done following the method described by Pan *et al*. (1991). Root and leaf samples (1 g each) were crushed in liquid nitrogen and extracted using 5ml of chilled 0.05 (M) sodium acetate buffer (pH-5) by grinding at

4ºC using mortar and pestle. The extract was then centrifuged at 10000 rpm for 15 min at 4ºC and the supernatant was used as crude enzyme extract. Estimation of the ß-1, 3-glucanase was done by following the Laminarin-dinitro salicylate method (Pan *et al*. 1991). The crude enzyme extract of 62.5 µl was added to 62.5 µl of laminarin (4%) and then incubated at 40ºC for 10 minutes. The reaction was stopped by adding 375 µl dinitrosalicylic reagent and heating for 5 min in boiling water bath. The resulting colored solution was diluted with 4.5 ml of water, vortexed and absorbance was taken at 500 nm. The blank was the crude enzyme preparation mixed with laminarin with zero-time incubation. The enzyme activity was expressed as µg glucose released min^{-1} g^{-1} fresh tissues.

***Chitinase* (*E.C. 3.2.1.14*)**

Extraction of chitinase was done by following the method described by Boller and Mauch (1988) with modifications. Root and leaf samples (1g each) from mandarin plants were crushed in liquid nitrogen and extracted using 5ml of chilled 0.1M sodium citrate buffer (pH5). The homogenate was centrifuged for 10minutes at 12,000 rpm and the supernatant was used as enzyme source. Chitinase activity was measured according to the method described by (Boller and Mauch, 1988). The assay mixture consisted of 10 µl Na-acetate buffer (1M) pH 4, 0.4 ml of enzyme solution, 0.1ml of colloidal chitin (1mg). Colloidal chitin was prepared as per the method of. After 2h of incubation at 37ºC the reaction was stopped by centrifugation at 10,000 g for 3 minutes. An aliquot of supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 µl of potassium phosphate buffer (1M) pH7.1 and incubated with 20 µl of (3%w/v) desalted snail gut enzyme Helicase (Sigma) for 1h. After 1h, the pH of the reaction mixture was brought to 8.9 by addition of 70 µl of sodium borate buffer (1M) pH 9.8. The mixture was incubated in a boiling water bath for 3 minutes and then rapidly cooled in an ice water bath. After addition of 2 ml of DMAB (ñ-dimethyl-aminobenzal-dehyde) reagent the mixture was incubated for 20 min at 37 ºC. Therefore absorbance value at 585 nm was taken UV-VIS spectrophotometrically. N-acetyl glucosamine (GlcNAc) was used as standard. The enzyme activity was expressed as ìg GLcNAc min^{-1} mg^{-1} fresh tissues.

***Phenylalanine ammonia lyase* (*E.C. 4.3.1.5*)**

Extraction of PAL was done by following the method described by Chakraborty *et al*., (1993) with modifications. Root and leaf samples (1 g each) were crushed in0.1M sodium borate buffer pH 8.8 (5ml/gm) with 2mM of ß-mercaptoethanol in ice cold temperature. The slurry was centrifuged in 15,000 rpm for 20 min at 4ºC. Supernatant was collected and after recording its volume, was immediately used for assay or stored at-20ºC. Phenylalanine ammonia lyase activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The reaction mixture contained 0.3 ml of 300 µM sodium borate (pH 8.8), 0.3 ml of 30 µM L- phenylalanine and 0.5 ml of supernatant in a total volume of 3 ml. Following incubation for 1 h at 40 ºC the absorbance at 290 nm was read against a blank without the enzyme in the assay mixture. The enzyme activity was expressed as µg cinnamic acid produced min^{-1} g^{-1} fresh weight of tissues.

***Peroxidase* (*E.C.1.11.1.7*)**

Plant tissues were macerated to powder in liquid nitrogen and extracted in 0.1 M sodium borate buffer (pH 8.8) containing 2 mM ß mercaptoethanol under ice cold conditions.Peroxidase activity was assayed by monitoring the oxidation of O-dianisidine in the presence of H_2O_2 and expressed as the increase in absorbance at 460 nm g^{-1} tissue/ min^{-1} (Chakraborty *et al.* 1993). Extraction of PAL (E.C. 4.3.1.5) was also done by following the method of Chakraborty *et al.* (1993), and the enzyme activity at 290 nm was expressed as µg cinnamic acid produced in 1 min g^{-1} fresh weight of tissues.

Immunodetection of pathogen and defense enzymes

Immunodetection of pathogen in mandarin root tissues following challenge inoculation with *F. oxysporum* (UI) were also confirmed following indirect immunofluoresce test as described by Chakraborty (2021) using PAb of *F. oxysporum* and labelled with FITC conjugates. Besides, purified lgG raised against â,1-3-glucanase and chitinase were used separately and labeled with goat antisera specific to rabbit globulins conjugated with Fluorescein isothiocyanate (FITC) for indirect immunofluorescence study of cellular location in SMC treated root tissue of mandarin plants following the protocol as described by Chakraborty (2021). Observations were made using a Biomed microscope (Leitz) equipped with I-3 filter block ideal for FITC fluorescence under UV light in the

dark. Photographs were taken by Moticam Pro 285B.

RESULTS

The mycelial growth of *F. oxysporum* was partially inhibited *in vitro* by *A. bisporus* and cold sterilized SMC extract of *A. bisporus* (Table 1, Fig.1). Toxin secretion by *F. oxysporum* in PDA medium mixed with cold sterilized SMC of *A. bisporus* was evident (Fig 1C) in relation to medium control (Fig 1A) and medium mixed with steam sterilized SMC of *A. bisporus* (Fig 1B). Zone of inhibition at line of contact between cold sterilized SMC with *F. oxysporum* and *A. bisporus* was also evident (Fig 1 D).

Effect of SMC on seedling growth of mandarin

The effect of SMC on growth parameters was evaluated in terms of per cent increase in height and leaf number over healthy plants after one and two months of treatment. Results (Fig 2) revealed plant growth improvement such as enhancement of number of leaves (Fig 2A) and plant height (Fig 2B) following application of SMC of *A. bisporus* in rhizosphere.

Effect of SMC treatment on root rot disease development

Rhizosphere of mandarin was amended with SMC prior to challenge inoculation with *F. oxysporum*.

Table 1. *In vitro* antagonistic tests of SMC extract and *A. bisporus* against root rot pathogen (*F. oxysporum*)

Interacting microorganisms	Diameter of fungal colony (mm)	Inhibition (%)
F. oxysporum	10.3± 0.24	0
F. oxyspor um + SMC (*cold sterilized crude extract*)	5.1± 0.23	50.5
F. oxysporum + SMC autoclaved extract	7.1± 0.21	31.06
F. oxysporum + Agaricus bisporus	4.6± 0.20	55.3

Values are average of three replicate experiments. ± standared error

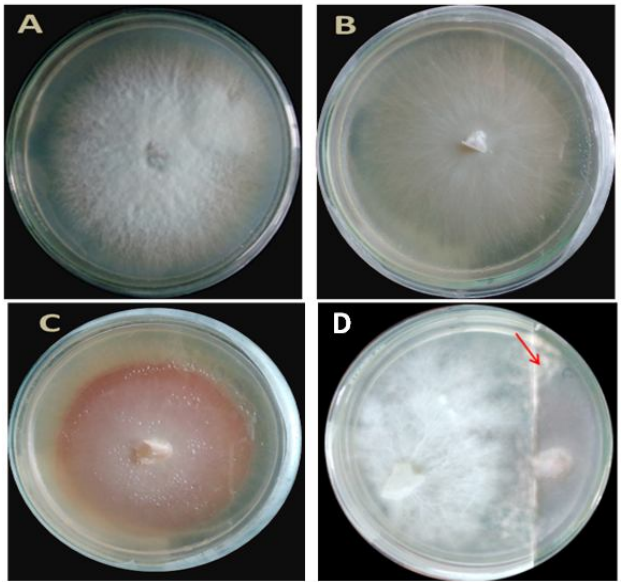

Fig.1: *In vitro* evaluation of SMC extract and *A. bisporus* against fungal pathogen. *F. oxysporum* grown on: (A) PDA medium (control) (B) Steam sterized SMC extract, (C) Cold sterilzed SMC extract and (D) *F. oxysporum* paired with *A. bisporus* in presence of cold sterilized SMC extract.

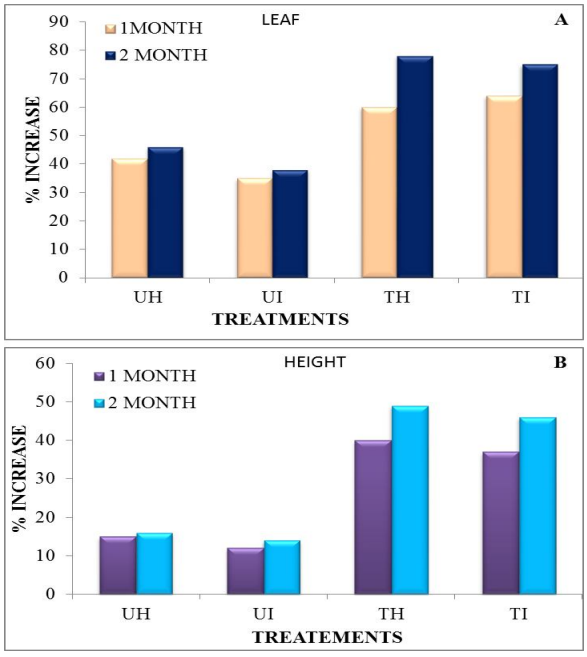

Fig. 2: Effect on growth of mandarin seedlings following application of Spent mushroom compost (SMC) of *Agaricus bisporus* in rhizosphere

Table 2. Root rot disease index of *Citrus reticulata* following treatment with spent mushroom compost of *Agaricus bisporus*

Days after inoculation	Disease index			
	Pre-treated with SMC and inoculated with pathogen			
	F. oxysporum (UI)	*F. oxysporum* + SMC (TI)	Normal Control (UH)	Treated Healthy (TH)
15	3.5 ± 0.116	2.7 ± 0.078	0	0
30	4.2 ± 0.102	2.3 ± 0.121	0	0
45	6.1 ± 0.096	1.7 ± 0.156	0	0

Values are average of 20 replicates, ± standard error

Rot index: 0- no symptoms; 1- small roots turn brownish and start rotting; 2- leaves start withering, and 20-40% of roots turn brown; 3- leaves withered and 50% of roots affected; 4- shoot tips also start withering; 60-70% roots affected; 5- shoots withered with defoliation of lower withered leaves, 80% roots affected; 6- whole plants die, with upper withered leaves remaining attached; roots fully rotted.

Fig. 3 (A-F). Evaluation of SMC of *A. bisporus* on development of root rot of *Citrus reticulata* against *F. oxysporum.* (A) Healthy plant (B) Root rot mediated wilting symptom (C) Untreated healthy (D) Untreated inoculated (E) SMC treated healthy plants (F) SMC treated plants after challenge inoculation with *F. oxysporum*

Root rot disease development was determined following application of SMC after 15, 30 and 45 days of inoculation. Results (Table 2) revealed that SMC could reduce root rot disease significantly (Table 2, Fig.3).

Biochemical changes in defense enzymes following SMC treatment

Significant biochemical changes were observed on application of SMC of *A. bisporus* on rhizosphere of mandarin plants prior to inoculation with pathogen. Experiments were conducted to assess the effect of SMC on accumulation of defense enzymes in mandarin root and leaf tissue. Multifold increase in the activities of chitinase, â-1,3-glucanase, peroxidase and phenylalanine ammonia lyase in roots as well as in leaf of mandarin plants were observed after application of SMC followed by inoculation with *F. oxysporum* (Figs. 4 & 5) Overall results show that the defense enzyme activities were higher in leaves than in the root.

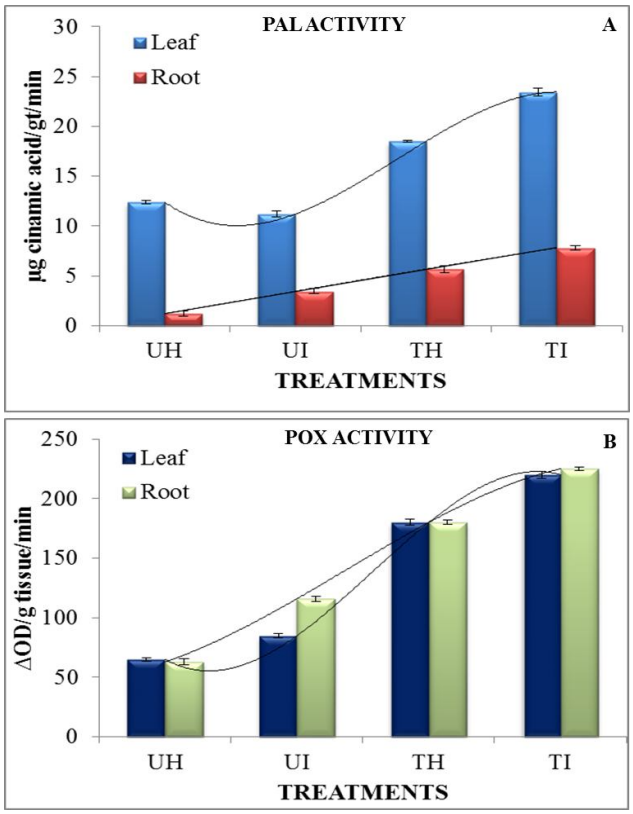

Fig. 4 (A&B). (A) Phenyl Ammonia Lyase (PAL) and (B) Peroxidase activities in root and leaf tissues of *Citrus reticulata* on application of SMC of *A. bisporus* following inoculation with *F. oxysporum*

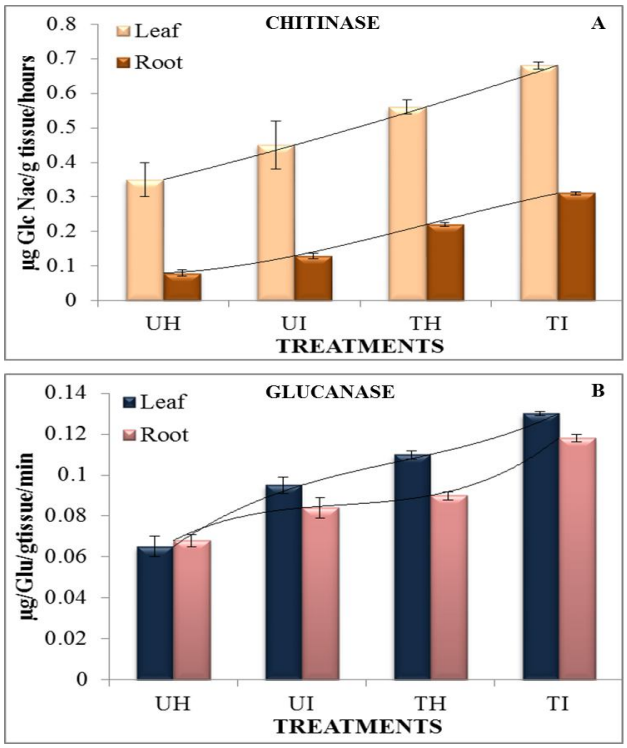

Fig 5(A&B). (A) Chitinase and (B) Glucanase activities in root and leaf tissues of *Citrus reticulata* on application of SMC of *A. bisporus* following inoculation with *F. oxysporum*

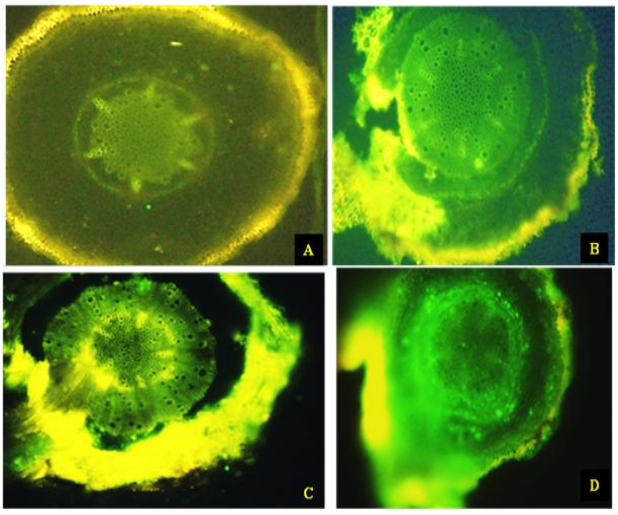

Fig. 6 (A-D): Indirect imunofluorescence of mandarin root tissue of (A) Healthy plant , (B-D) Untreated plant challenge inoculated with *F. oxysporum* after appearance of wilting symptom probed with PAbs of pathogen and labelled with FITC.

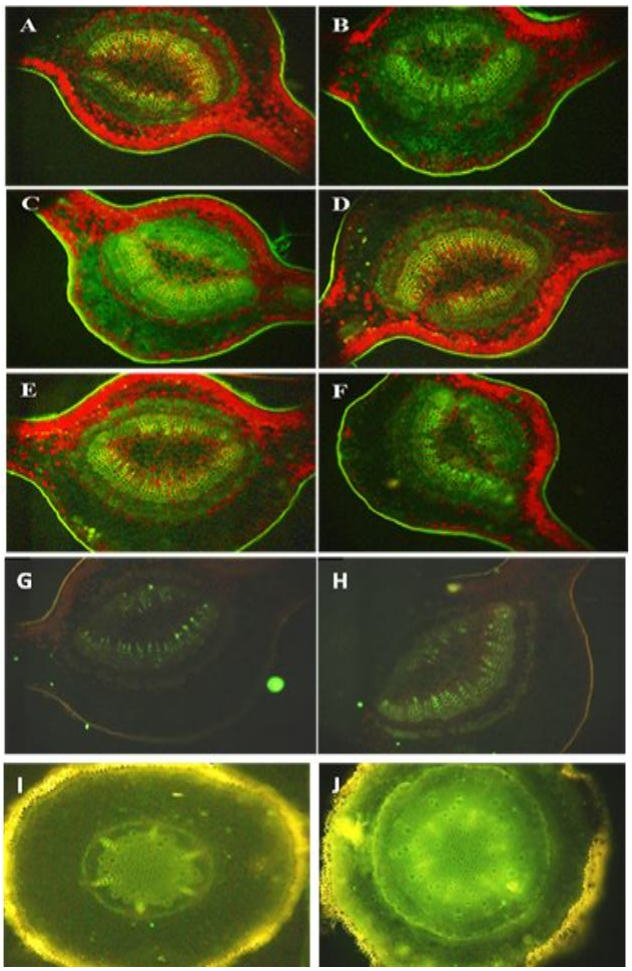

Fig. 7 (A-J): Cellular localization of â,1-3-glucanase in leaf (A-H) and root (I-J) tissue of *Citrus reticulata* following SMC treatment probed with PAbs of â,1-3-glucanase labelled with FITC.(A-D) SMC treated, (E&F) SMC treated inoculated with *F. oxysporum,* (G&H) untreated control and (I&J) SMC treated root inoculated with *F. oxysporum*

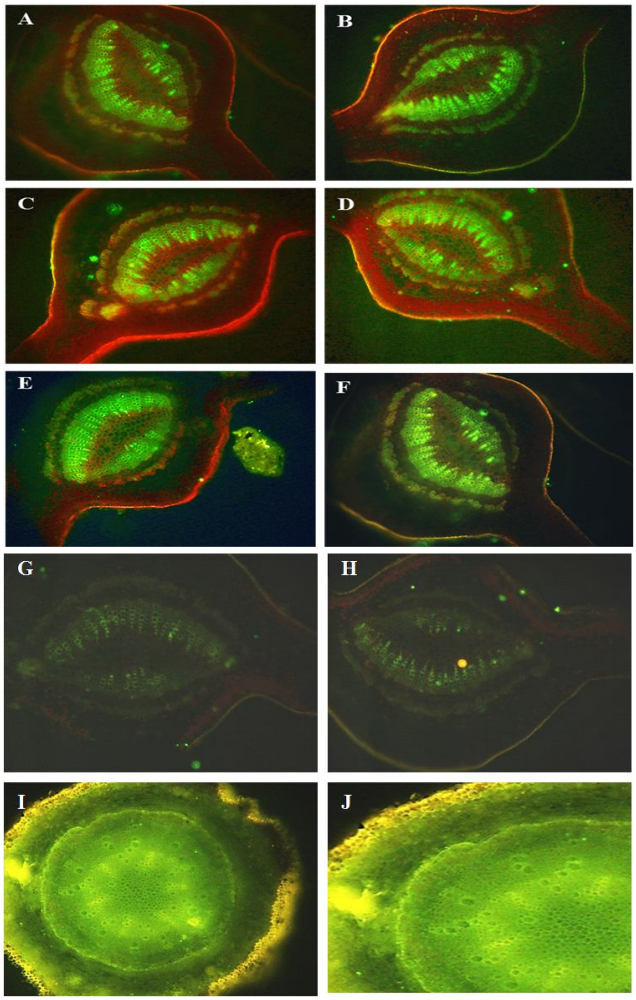

Fig. 8 (A-J): Cellular localization of chitinase in leaf (A-H) and root (I-J) tissue of *Citrus reticulata* following SMC treatment probed with PAbs of chitinase labelled with FITC. (A-D) SMC treated, (E&F) SMC treated inoculated with *F. oxysporum,* (G&H) untreated control and (I&J) SMC treated root inoculated with *F. oxysporum.*

Activities of all four enzymes, in both leaves and roots, were significantly ($p<0.01$) enhanced due to the SMC treatment (Figs. 4 and 5). PAL activity was more than double in leaves when compared to root (Fig 4A), whereas, peroxidase activity was almost similar in leaves and roots (Fig.4B). The chitinase activity was more in leaves in compared to root (Fig 5A) whereas â-1,3 glucanase activity was almost similar in both leaves and root (Fig 5B).

Detection of pathogen in root tissue of mandarin plant

Indirect immunofluorescence of *F. oxysporum* inoculated root tissues of mandarin plants using homologous polyclonal antibody of pathogen (*F. oxysporum*) and labelling with FITC conjugates showed bright apple green fluorescence in cortical as well as vascular tissues (Fig. 6 B-D). Healthy control root tissue when treated with normal serum followed by FITC showed auto-fluorescence in epidermal tissue and pith area (Fig 6A).

Immunolocalization of defense enzymes in root and leaf tissues of SMC treated mandarin plants following inoculation with F. oxysporum

Root rot incidence in mandarin plants was successfully reduced after application of SMC in the rhizosphere of mandarin saplings prior to pathogen challenge. Disease reduction was found to be brought about by enhanced activities of key defense enzymes like chitinase, glucanase, phenylalanine ammonia lyase and peroxidase which increased significantly after pathogen challenge. Cellular localization of chitinase in leaves and roots of citrus plants was determined following indirect immuno-fluorescence test using FITC binding and treatment with PAbs raised against chitinase and glucanase. Cellular localization of chitinase and glucanase in mandarin root and leaf and tissues were determined following an indirect immunofluorescence test using PAb raised against glucanse (Fig. 7 A-J) and chitinase (Fig. 8 A-J) separately labeled with FITC. Strong apple green fluorescence was observed in SMC treated leaf mainly in mesophyll tissues and cortical and vascular tissue in SMC teated root tissues following challenge inoculation with pathogen indicating induction of defense enzymes (chitinase and glucanase) in root and leaf tissues. Bright apple green fluorescence was considered as the positive reaction.

DISCUSSION

In the present study regarding the application of spent mushroom compost in crop system it was found that severity of root rots disease in *Citrus reticulata* caused by *F. oxysporum* was reduced by the treatment of SMC of *A. bisporus* in potted condition. Our study was supported by the result of Parada *et al*. (2012) where they used autoclaved water extract from spent mushroom substrate and autoclaved spent mushroom substrate of the edible mushrooms *Lyophyllum decastes* and *Pleurotus eryngii* to reduce powdery mildew diseases caused by *Podosphaera xanthii* and bacteria *Pseudomonas syringae* on cucumber plants. Kwak

et al. (2015) conducted as experiment in which they used spent substrate water extracts of edible mushrooms and promoted growth of pepper seedling as well as mycelial growth rate of *Phythopthora capsici* and *Fusarium oxysporum* was dramatically inhibited by 100% and 70% in vitro by using SMS extract. These results have supported our study and contributed support to conclude the SMS extract have dual effects that suppress plant disease and promote plant growth. In our study the mycelial growth of *F. oxysporum* was also inhibited by the mycelial phase of six mushroom as well as the by the SMC extract. Up to 52% inhibition of mycelial growth and sporulation by the SMC water extract was recorded. Suarez *et al*. (2012) studied *in vitro* control of *Fusarium* wilt using agro-industrial subproduct-based composts. Choi *et al.,* (2007) successfully reduced the basal stem rot of cactus caused by *F. oxysporum* using spent mushroom compost. Ayala *et al*. (2015) and Bastida *et al*. (2016) successfully studied comparative antibacterial activity of the spent substrate of *Pleurotus ostreatus* and *Lentinula edodes.* Antimicrobial activity of mushroom extract was also evaluated by Nehra *et al*. (2012). Spent mushroom substrate of oyster mushroom as biofertlizer for growth improvement of *Capsicum annum* has been demonstrated (Roy *et al*., 2015). Using spent mushroom compost, management strategies of wilt disease caused by *F. oxysporum* on tomato has also been developed (Hussein *et. al*. 2016). In the present investigation induced resistance in manadarin plants against *F. oxysporum* following SMC treatment was confirmed by immunolocalization of defense enzymes both in root and leaf tissues.The spent musroom comopost (SMC) left after final crop harvest of button mushroom is a matter of concern for all of us to utlize for agricultural productivity as well as it can also ameliorate the problem of solid waste disposal in the mushroom industry.

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