# Physiological changes in *Alternaria solani* treated with Nativo®75 WG

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**Abstract**

Early blight is an important disease in tomato. *Alternaria solani* is the causal organism of this disease, which produces brown to black spots with 'bull's eye' appearance on lower and mid-canopy leaves. Symptoms in tomato fruit are usually found associated with the stem end and shoulder and may expand in size. Fruit symptoms include a sunken, greenish-brown-black spot with concentric rings. Nativo®75 WG, mixture of Trifloxystrobin 25 %+ Tebuconazole 50% - 75 WG, acts very effectively against *Alternaria solani* by inhibiting both sterol biosynthesis as well as respiration. In the present work, we studied the invitro as well as invivo efficacy and effect on fungal physiology of a mixture of Trifloxystrobin 25 % + Tebuconazole 50% - 75 WG marketed as "Nativo®75 WG" by Bayer Crop Science Ltd. and compared this with standard broad spectrum fungicides commonly used for the control of *A. solani.* A 700 ppm concentration of Nativo®75 WG was significantly effective in control of early blight in the field. In vitro, a 140 x dilution was sufficient to check the growth rate and spore germination rate by up to 50%. The effects of Nativo®75 WG on *A. solani* include inhibition of growth rate and spore germination, and changes in enzyme activity and enzyme isomer patterns for superoxide dismutase, catalase, peroxidase, and α and β esterase formation.

**Key words:** *Alternaria solani*, oxidative stress, tebuconazol, trifloxystrodin.

## Introduction

Tomato (*Lycopersicon esculentum* Mill.) belongs to the family Solanaceae and is one of the most widely grown vegetables in the world. Among the vegetables, tomato ranks next to potato in world acreage and ranks first among the processing crops. In 2006, the total area under tomato was 4,615 thousand ha with production of 127,993 thousand tons and with a productivity of 27.6 tons/ha (Indian Horticulture database 2006). Diseases of tomato are caused by fungi, bacteria, viruses, nematodes and abiotic factors (Balanchard 1992). Among the fungal diseases, early blight, also known as target spot, caused by *Alternaria solani*, is one of the most serious diseases. The causal organism is air borne and soil inhabiting, and symptoms include leaf blight, collar rot of pedicel and fruit rot of tomato (Datar & Mayee 1981). The disease appears on leaves, stems, petiole, pedicel and fruits under favorable conditions (warm temperatures (e.g. 23-28°C), extended period of leaf wetness from frequent rain or overhead sprinkler irrigation) resulting in defoliation, drying off of the pedicel and premature fruit drop, thus causing loss from 50-86% in fruit yield (Mathur & Shekhawat 1986). Spores of the fungi are one of most important means of dissemination. It is well established that the tomato early blight fungus can survive on infected seeds for several months in mycelium form. However, it is still speculative whether the seed borne inoculum of *A. solani* serves as a source for triggering primary infection in the next season (Neergaard 1945). The ability of the pathogen to survive for extended periods in the diseased plant parts, soil and on alternative/collateral hosts in the absence of the main host determine the ability of the pathogen to persist (Moore & Thomas 1942; Basu 1971; Rands1917a). Spraying of broad spectrum fungicides such as Mancozeb and Captan has been recommended for the control of early blight of tomato by several workers (Ramakrishnan *et al.* 1971; Stevenson 1977). However, even with frequent applications, these have low persistence on foliage (Thind & Jyothi 1982) and control is inadequate. In the present work, we studied the *in-vitro* and *in-vivo* efficacy and effect on fungal physiology of Trifloxystrobin 25 % + Tebuconazole 50% - 75 WG (Nativo®75 WG, Bayer Crop Science Ltd.) as compared with the standard broad spectrum fungicides used for the control of *A. solani*. Primarily, we screened the efficacy of Nativo®75 WG in field conditions. This was followed by testing of invitro bio-efficacy at different doses. Thereafter we investigated the physiological changes occurring in *A. solani* in response to fungicidal shock at effective concentration 50 ($EC_{50}$).

## Materials and Methods

An experimental trial was conducted to evaluate the field efficacy of Nativo®75 WG for two consecutive years (2009 & 2010) in C-Block farm, B.C.K.V, Kalyani, West Bengal, India, maintaining all standard and recommended agronomic practices including tillage, spacing, manuring, irrigation and insect control for cultivation of the crop. The test fungicides (Table 1) were applied as foliar sprays with an ASPEE sprayer. The test fungicides were mixed with 500 litres of water/ha as per manufacturers recommendations. The first

**Table 1.** Treatment details for Bio-efficacy field trials.

| Treatment | Dose (gm active ingredient/ha) | Dose (g Product/ha) |
|---|---|---|
| **Untreated Control** | - | - |
| **Tryfloxystrobin + Tebuconazole 75WG** | 62.5+125 | 250 |
| **Tryfloxystrobin + Tebuconazole 75WG** | 75+150 | 300 |
| **Tryfloxystrobin + Tebuconazole 75WG** | 87.5+175 | 350 |
| **Flint** | 87.5 | 175 |
| **Folicur** | 175 | 700 |
| **Folicur** | 187.5 | 750 |
| **Mancozeb** | 937.5 | 1250 |

application was 45 days after transplanting (DAT) when the initial symptoms (leaf necrosis) of the disease appeared on the tomato plants. This was followed by two more sprays at 10 day intervals. Four observations of disease severity were recorded one day before each spraying. In addition yield, plant height and fruit characteristics (length, girth, total soluble solids (TSS)) were recorded. Disease scoring was done on a 0 – 4 scale (Mayee & Datar 1981). The percent disease index (PDI) was calculated by the following formula:

PDI = [Sum of all numerical ratings / (Total no. of observations x Maximum rating scale)] x100

Nativo®75 WG was tested for in vitro efficacy against *A. solani* isolated from diseased tomato leaves. The *A. solani* isolates were grown on potato dextrose agar (PDA, Hi-media) slants incubated at 24±2°C. Nativo®75 WG was evaluated at 0.1, 0.5, 1, 3, 5, 7, 10 and 20 ppm in vitro against *A. solani* following the 'poisoned food technique' (Nene & Thapliyal, 1993). The fungicides (marketed product) were added after filter sterilization to sterile PDA medium kept separately in conical flasks, to give final concentrations of 0.1, 0.5, 1, 3, 5, 7, 10 and 20 ppm. After thorough mixing of fungicide, the media were poured into sterilized Petri plates. Plates containing PDA without fungicide served as controls. The plates were inoculated with 5 mm mycelial discs of ten day old PDA cultures of *A. solani* and incubated at 24±2°C. The experiment was laid out in complete randomized design with three replications for each treatment. Colony diameter was measured at 24 hr intervals and percent growth inhibition and $EC_{50}$ calculated by comparing the growth rate between

**Table 2.** Field trial for efficacy of Tryfloxystrobin 25% + Tebuconazole 50% (Nativo 75 WG) against early blight of tomato (2009).

| Treatments | g active ingredient/ha | Disease severity % (Arc-Sine transformed value) | | | | Disease control % | Yield tonne/ha |
|---|---|---|---|---|---|---|---|
| | | Days after sowing: | | | | | |
| | | 44 | 54 | 64 | 74 | | |
| **Untreated Control** | - | 2.75 (9.55) | 19.50 (26.21) | 33.4 (35.30) | 62.7 (52.36) | - | 25.25 |
| **Tryfloxystrobin + Tebuconazole 75WG** | 62.5 +125 | 2.25 (8.63) | 14.10 (22.06) | 25.3 (30.20) | 45.2 (42.25) | 27.91 | 27.64 |
| **Tryfloxystrobin + Tebuconazole 75WG** | 75 + 150 | 3.50 (10.78) | 10.50 (18.91) | 20.8 (27.13) | 38.2 (38.17) | 39.07 | 29.31 |
| **Tryfloxystrobin + Tebuconazole 75WG** | 87.5 +175 | 2.00 (8.13) | 6.5 (14.77) | 13.7 (21.72) | 26.2 (30.79) | 58.21 | 32.08 |
| **Flint** | 87.5 | 1.50 (7.03) | 10.20 (18.63) | 18.9 (25.77) | 37.1 (37.52) | 40.83 | 27.58 |
| **Folicur (250 EC)** | 175 | 2.70 (9.46) | 11.10 (19.46) | 21.4 (27.56) | 39.2 (38.76) | 37.48 | 26.90 |
| **Folicur (250 EC)** | 187.5 | 3.80 (11.24) | 9.10 (17.56) | 16.9 (24.27) | 34.6 (36.03) | 44.82 | 29.28 |
| **Mancozeb 75WP** | 937.5 | 4.50 (12.25) | 12.50 (20.70) | 22.4 (28.25) | 41.8 (40.28) | 33.33 | 26.81 |
| **Standard error of mean (±)** | - | 0.234 | 0.908 | 1.276 | 0.936 | - | 0.033 |
| **Critical Difference at 5%** | - | 0.711 | 2.752 | 3.868 | 2.838 | - | 0.101 |

**Table 3.** Field trial for efficacy of Tryfloxystrobin 25% + Tebuconazole 50% (Nativo 75 WG) against early blight of tomato (2010).

| Treatments | g active ingredient/ha | Disease severity % (Arc-Sine transformed value) Days after sowing: | | | | Disease control % | Yield tonne/ ha |
|---|---|---|---|---|---|---|---|
| | | 44 | 54 | 64 | 74 | | |
| **Untreated Control** | | 1.25 (6.42) | 15.2 (22.95) | 28.7 (32.39) | 58.9 (50.13) | - | 29.5 |
| **Tryfloxystrobin + Tebuconazole 75WG** | 62.5+125 | 0.75 (4.97) | 12.4 (20.62) | 21.4 (27.56) | 43.6 (41.32) | 26.0 | 32.70 |
| **Tryfloxystrobin + Tebuconazole 75WG** | 75+150 | 1 (5.74) | 8.9 (17.36) | 16.8 (24.20) | 35.2 (36.39) | 40.2 | 34.37 |
| **Tryfloxystrobin + Tebuconazole 75WG** | 87.5+175 | 1.75 (7.60) | 4.9 (12.79) | 11.7 (20.00) | 23.3 (28.86) | 60.4 | 37.14 |
| **Trifloxystrobin 50 WG (Flint 50 WG)** | 87.5 | 2.25 (8.63) | 7.9 (16.32) | 14.5 (22.38) | 32.6 (34.82) | 44.7 | 32.64 |
| **Tebuconazole 250 EC (Folicur 250 EC)** | 175 | 1.5 (7.03) | 9.2 (17.66) | 17.6 (24.80) | 35.9 (36.81) | 39.0 | 31.96 |
| **Tebuconazole 250 EC (Folicur 250 EC)** | 187.5 | 0.75 (4.97) | 7.3 (15.68) | 13.7 (21.72) | 31.4 (34.08) | 46.7 | 33.34 |
| **Mancozeb 75 WP** | 937.5 | 1.5 (7.03) | 11.8 (20.09) | 20.6 (26.99) | 40.1 (39.29) | 31.9 | 31.87 |
| **Standard error of mean (±)** | | 0.088 | 0.895 | 0.787 | 1.41 | - | 0.347 |
| **Critical Difference at 5%** | | 0.268 | 2.72 | 2.39 | 4.29 | - | 1.06 |

control and treated plates. After 10 days of growth, two 5 mm mycelial discs were cut from sporulating zone and suspended in 1 ml sterile water and homogenized to make spore suspensions, which were examined by light microscope (400 X) for conidial size and number of septa.

To evaluate inhibition of spore germination after Nativo®75 WG treatment, an *A. solani* spore suspension ($10^5$ spores $ml^{-1}$) was prepared from a ten day-old fungal culture. The spore suspension (0.1 ml) was placed in a cavity glass slide containing 0.1 ml of each concentration of fungicide. These slides were kept in moist chambers prepared by placing a fold of water soaked filter paper in both base and lid of Petri-plates and the plates were incubated at 24±2°C for 12 hrs continuous light (4 x $10^3$ lux). Each treatment was replicated five times. The percent spore germination (SGI%) was recorded using the formula given by Kiraly *et al.* (1974). Percent spore germination inhibition and effective concentration 50 (EC50) were determined by comparing spore germination percentage between control and treated slides.

Percent spore germination = (No. of spores germinated / Total no. of spores examined) x 100

The efficacy of each treatment was estimated as follows (Maouni et al, 2007):

**Table 4.** Field trial for efficacy of Tryfloxystrobin 25% + Tebuconazole 50% (Nativo 75 WG) - tomato morphological parameters.

| Treatment | Plant height (cm) | Fruit length (cm) | Fruit Girth (cm) | Total Soluble Solid |
|---|---|---|---|---|
| **Untreated Control** | 80.00 | 2.59 | 3.10 | 3.49 |
| **Tryfloxystrobin + Tebuconazole 75 WG[1]** | 87.00 | 2.98 | 3.32 | 3.84 |
| **Tryfloxystrobin + Tebuconazole 75 WG[2]** | 107.00 | 3.39 | 4.08 | 4.42 |
| **Tryfloxystrobin + Tebuconazole 75 WG[3]** | 117.53 | 4.44 | 4.96 | 5.00 |
| **Trifloxystrobin 50 WG (Flint 50 WG)** | 110.27 | 3.57 | 4.26 | 4.62 |
| **Tebuconazole 250 EC (Folicur 250 EC)** | 97.67 | 3.21 | 3.80 | 4.32 |
| **Tebuconazole 250 EC (Folicur 250 EC)** | 113.67 | 3.95 | 4.47 | 4.73 |
| **Mancozeb 75 WP** | 94.33 | 3.14 | 3.59 | 4.01 |
| **Critical Difference at 5 %** | 3.91 | 0.077 | 0.633 | 0.325 |
| **Standard error of mean (±)** | 1.34 | 0.026 | 0.217 | 0.111 |

Dose: [1] 62.5+125, [2] 75+150 and [3] 87.5+175.

**Table 5.** *A. solani* conidial parameters in control and 5 ppm Nativo treatments.

| Conidial parameters | Control | 5 ppm Nativo ®75 WG | Critical Difference at 5 % | Standard error of mean (±) |
|---|---|---|---|---|
| **No. of horizontal septa** | 3.6 | 4 | 0.351 | 0.121 |
| **No. of vertical septa** | 0.5 | 0.79 | 0.321 | 0.111 |
| **No. of oblique septa** | 0.14 | 0.21 | 0.325 | 0.112 |
| **Conidial length (µm)** | 33.26 | 25.65 | 2.773 | 0.956 |
| **Length of conidia without beak (µm)** | 27.22 | 22.93 | 2.7 | 0.931 |
| **Beak length (µm)** | 6.04 | 2.72 | 1.468 | 0.506 |
| **Width of conidia (µm)** | 14.26 | 9.37 | 1.5 | 0.517 |

$$E\ (\%) = [(X - X_i)/ X] \times 100$$

X = estimation of germination, or growth in a medium without fungicide (Control). $X_i$ = estimation of germination, growth or sporulation in a medium with fungicide.

**Preparation of fungal tissue in broth culture**

The fungus was grown for 12 days in potato dextrose broth (PDB, Hi-media) containing 5 ppm fungicide at static condition. Mycelial mats were harvested at 4 day intervals for biochemical analysis. Broth without fungicide served as a control. Broths were inoculated with four 5 mm mycelial discs taken from the active growing regions of the respective treatments (control and 5 ppm fungicide). The fungal mats were harvested using Whatman No. 1 filter paper; washed with sterile double distilled water three times and then the fungal tissue was wrapped in brown paper and kept in a 500°C oven overnight to dry.

**Extraction and electrophoresis of isoenzymes**

Freshly harvested fungal tissue was crushed with Tris-Citrate buffer (pH-7.8) and Na-P buffer (pH-7) for esterase (EST) and for superoxide dismutase (SOD), peroxidase (PO), catalase (CAT) isomer detection, respectively. Electrophoresis of EST isoenzymes was done using an 11% polyacrylamide gel, and PO, SOD and CAT used a 10% polyacrylamide gel, according to the method of Kahler & Allard (1970). The Relative front (Rf) of the migrating bands was calculate as Distance migrated by the isomer band / Length of the gel subjected to electrophoresis.

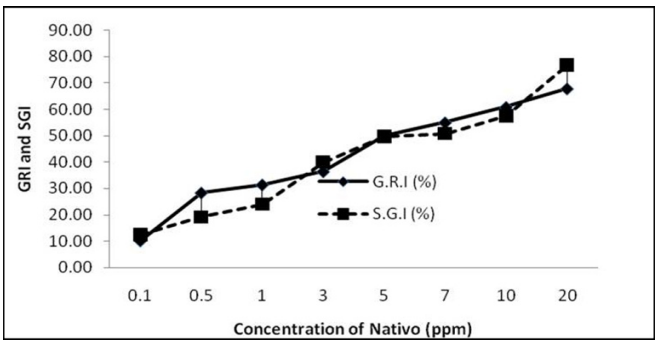


**Fig. 1** Growth rate and percent spore germination inhibition of *A. solani* by different doses of Nativo treatment, GRI = Growth Rate Inhibition; SGI = Spore Germination Inhibition.

**Staining of isoenzyme gel**

α-esterase and β-esterase were stained according to Mostafa et al (2003). Peroxidase (PO) (EC 1.11.1.7) was stained using orthodinisidin (1 mg $ml^{-1}$ methanol) with 0.2 M hydrogen peroxide. The gel was incubated in darkness until brownish orange bands appeared (Malik & Singh 1980). SOD gel staining was done according to Madamanchi et al (1994). The CAT (EC:1.11.1.16) gel was incubated in 3.3 mM hydrogen peroxide for 20 min in darkness, washed with distilled water and treated with 0.1 % ferric chloride and 0.1 % potassium permanganate solution (1:1 V/V) for 15 min at which time white bands appeared on a dark green background (Woodbury et al 1971).

**Spectrophotometric assay**

Spectrophotometric assay of PO, SOD, CAT, EST was done by modifying the method of Malik & Singh (1980), Beauchamp & Fridovich (1971), Woodbury *et al.* (1971), and Mostafa *et al.* (2003) respectively, and was expressed as enzyme activity (EA) g of $tissue^{-1}$ $min^{-1}$. Assays of phenol, total carbohydrate and total protein were done according to Malick & Singh (1980), Hedge & Hofreiter (1962), and Lowry *et al.* (1951) respectively, and expressed as mg $g^{-1}$ tissue.

**Statistical Analysis**

The data collected during these investigations were analyzed in the Duncan multiple range test (DMRT)[p = 0.05] of the Univariate ANOVA model in SPSS statistical tool 10.0.

## Results

**In vivo efficacy of Nativo**

All treatments significantly reduced early blight disease disease severity compared to the untreated plot. However, Tryfloxystrobin 25% + Tebuconazole 50% (Nativo®75 WG) @ 87.5+175 g $a.i.ha^{-1}$ proved more effective compared to Folicur, Flint and Mancozeb. Disease intensity was reduced by 58% (2009) and 60% (2010). Tryfloxystrobin 25% + Tebuconazole 50% (Nativo®75 WG) @ 87.5+175 g $a.i.ha^{-1}$ also proved to be the more effective showing the lowest disease index (26% in 2009 and 23% in 2010) for controlling early blight (Tables 2, 3). The highest yield of tomato (32 t $ha^{-1}$ in 2009 and 37 t $ha^{-1}$ in 2010) was obtained in the Tryfloxystrobin 25% + Tebuconazole 50% (Nativo®75 WG) @ 87.5+175 g a.i. $ha^{-1}$ treated plot as compared

to other fungicide-treated and untreated plots. The maximum percent increase of yield obtained in Tryfloxystrobin 25% + Tebuconazole 50% (Nativo®75 WG) @ 87.5+175 g a.i.ha$^{-1}$ treated plots were 27% in 2009 and 26% in 2010. In addition, plant height (117.53 cm), fruit length (4.44 cm), fruit girth (4.96 cm) and total soluble solids (TSS) of fruits (5.00) significantly improved in Tryfloxystrobin 25% + Tebuconazole 50% (Nativo®75 WG) @ 87.5+175 g a.i. ha$^{-1}$ treatment compared with the other fungicides tested in this experiment (Table 4).

**In vitro Efficacy**

It was observed that at 20 ppm Nativo ®75 WG the growth rate inhibition percent (GRI %) of *A. solani* was 68% (Fig. 1) while concentrations above this resulted in cessation of growth (data not shown). Hence, concentrations below 20 ppm were selected for determination of $EC_{50}$. Every concentration of fungicide tested significantly reduced the growth rate and spore germination at every incubation interval. From spore germination inhibition data, the $EC_{50}$ was 5 ppm (Fig. 1). Further it was observed that there was a significant reduction in conidial size from above 5 ppm concentration of the fungicide compared to the untreated control however no effect was recorded on the numbers of septa in conidia at different concentrations (Table 5).

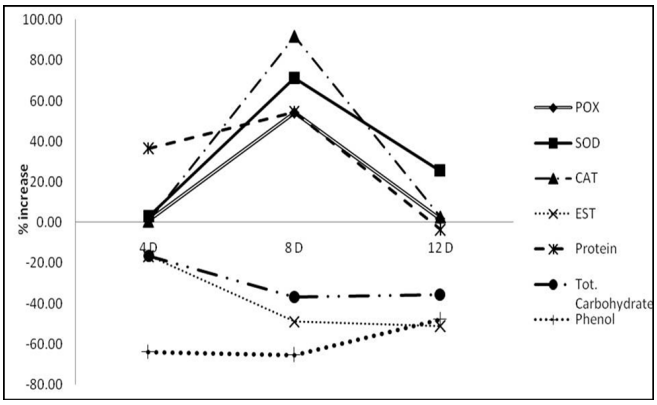


**Fig. 2** Percent increase of different biochemical parameters after different days of treatment. POX = Peroxidase, SOD = Superoxide dismutase, CAT = Catalase, EST = α Esterase.

**Estimation of bio-molecules and assay of enzyme activity**

Total protein, carbohydrate and phenol content in fungal tissue were altered with 5 ppm concentration of the fungicide compared with the control. Total protein content increased significantly on the 4$^{th}$ and 8$^{th}$ days of incubation and decreased after 12$^{th}$ day of treatment, while the maximum increase was observed on the 8$^{th}$ day (55 %). Total carbohydrate content decreased significantly on all days (4$^{th}$, 8$^{th}$ and 12$^{th}$) of incubation; however the maximum decrease of 37% was recorded

**Table 6.** Estimation of different bio-molecules after incubation of fungal tissue with fungicide.

| | Total Protein (mg. g$^{-1}$ tissue) | | | Total Carbohydrate (mg. g$^{-1}$ tissue) | | | Total Phenol (mg. g$^{-1}$ tissue) | | |
|---|---|---|---|---|---|---|---|---|---|
| | 4 D * | 8 D * | 12 D * | 4 D * | 8 D * | 12 D * | 4 D | 8 D * | 12 D |
| **Control** | 200.66 | 261.50 | 208.26 | 59.17 | 58.42 | 62.70 | 0.28 | 0.32 | 0.38 |
| **5 ppm Nativo ®75 WG** | 274.17 | 404.24 | 200.85 | 21.39 | 20.25 | 32.77 | 0.23 | 0.20 | 0.25 |
| **Critical Difference at 5 %** | 1.69 | 1.32 | 1.89 | 0.42 | 0.44 | 1.73 | 0.05 | 0.50 | 0.13 |
| **Standard error of mean (±)** | 0.38 | 0.29 | 0.42 | 0.09 | 0.10 | 0.38 | 0.01 | 0.01 | 0.03 |

* Significant at 0.05%.

**Table 7.** Activity of different enzymes after incubation of fungal tissue with fungicide.

| | PO (Enzyme Activity $^{-1}$ g tisssue$^{-1}$ m$^{-1}$) | | | SOD (Enzyme Activity $^{-1}$ g tissue$^{-1}$ m$^{-1}$) | | | CAT (Enzyme Activity $^{-1}$ g tissue$^{-1}$ m$^{-1}$) | | | EST (Enzyme Activity $^{-1}$ g tissue$^{-1}$ m$^{-1}$) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | **4 D *** | **8 D *** | **12 D *** | **4 D *** | **8 D *** | **12 D *** | **4 D *** | **8 D *** | **12 D *** | **4 D** | **8 D *** | **12 D** |
| **Control** | 1.044 | 1.073 | 1.231 | 1.415 | 1.495 | 1.659 | 1.005 | 1.084 | 1.055 | 1.655 | 1.647 | 1.492 |
| **5 ppm Nativo ®75 WG** | 1.053 | 1.654 | 1.252 | 1.455 | 2.560 | 2.082 | 1.012 | 2.078 | 1.085 | 1.421 | 1.105 | 0.986 |
| **Critical Difference at 5 %** | 0.125 | 0.125 | 0.217 | 0.106 | 0.373 | 0.779 | 0.047 | 0.116 | 0.082 | 0.474 | 0.585 | 0.335 |
| **Standard error of mean (±)** | 0.028 | 0.028 | 0.048 | 0.024 | 0.249 | 0.173 | 0.011 | 0.026 | 0.018 | 0.105 | 0.130 | 0.075 |

* Significant at 0.05%.

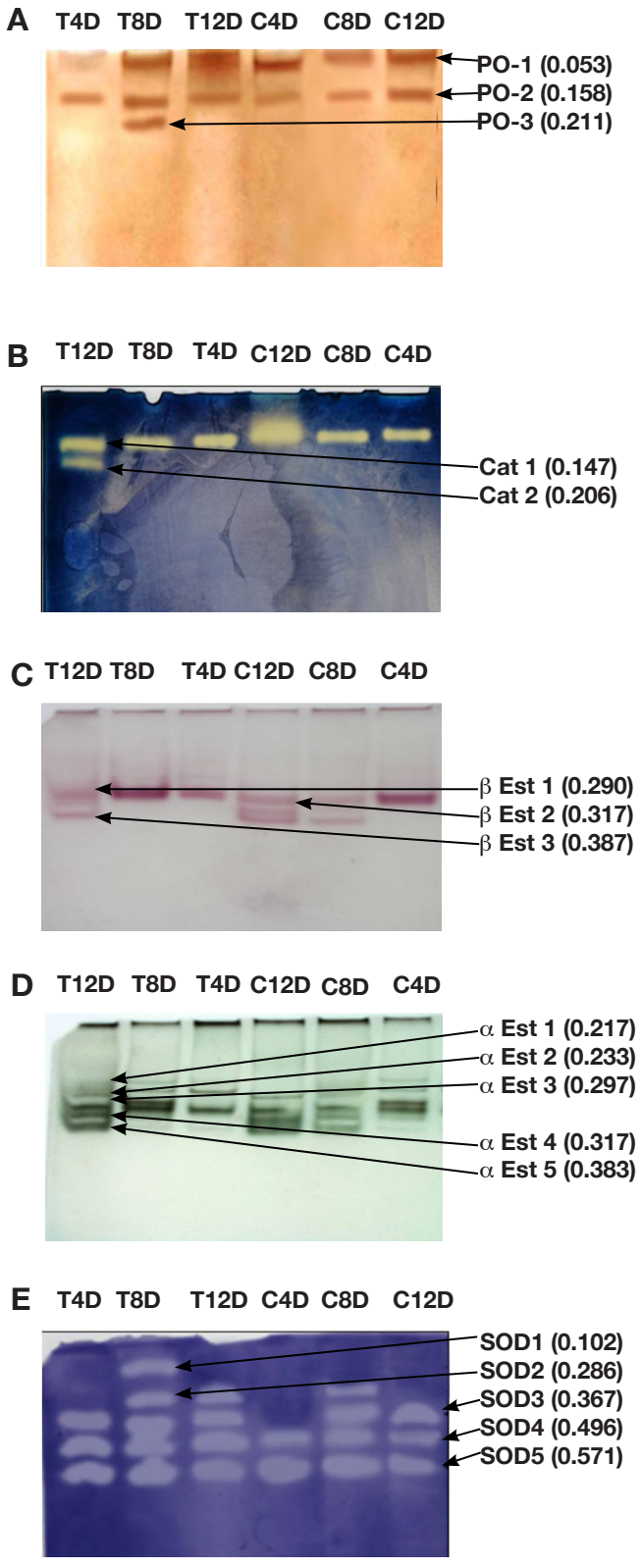


**Fig. 3** Native PAGE of fungal isozymes. **A** Peroxidase; **B** Catalase; **C** β esterase; **D** α esterase; **E** Superoxide dismutase (Rf values). Lanes: T12 D =12 days after fungicidal treatment; T8 D = 8 days after fungicidal treatment; T4 D = 4 days after fungicidal treatment; C12 D = Untreated fungal tissue after 12 days; C8 D = Untreated fungal tissue after 8 days; C4 D = Untreated fungal tissue after 4 days.

on the 8th day. However, in the case of total phenol, a steady decrease was noticed from the 4th up to the 12th day after treatment compared with the control, but the decrease was only significant (65%) on the 8th day (Table 6).

The PO, SOD, CAT and EST enzyme activities in the fungal tissue also changed after the treatment with the fungicide at 5 ppm (Table 7). The PO, SOD and CAT activities gradually increased up to 8th day after treatment. A significant increase was recorded on the 8th day after treatment (54 %, 71 % and 92 % respectively) (Fig. 2) and a new isomer PO-3 (Rf 0.211) appeared on the 8th day (Fig. 3 A). Similarly, a new CAT isomer CAT-1 (Rf 0.206) appeared on 12th day of treatment compared with the control (Fig. 3 B). EST activity recorded a steady decrease and the maximum significant decrease (51%) was recorded on the 12th day after treatment as compared with untreated 12 day old fungal tissue (Table 7). A new β-EST isomer β-Est-3 (Rf 0.387) was induced on 8th day of treatment as compared to control though there were no significant changes in isomer formation pattern in α-EST (Fig. 3 C&D). Native PAGE revealed the appearance of one new isomer, SOD-3 (Rf 0.367), on the 4th day and another, SOD-1 (Rf 0.102) on the 8th day of treated fungal tissue (Fig. 3E).

## Discussion

It is evident from the present work that all the fungicidal treatments significantly reduced early blight disease and increased the yield of tomato. However, compared with the other fungicides used in this experiment Tryfloxystrobin 25% + Tebuconazole 50% (Nativo®75 WG) @ 87.5+175 g a.i. $ha^{-1}$ (700 ppm) exhibited broad spectrum activity against early blight diseases of tomato with respect to minimization the disease severity, increasing the yield and other qualitative characteristics of the crop. As a result, different concentrations of the fungicide (Nativo®75 WG) were considered for bio-efficacy and biochemical testing. In the case of invitro bio-efficacy, it was evident from our work that Nativo®75 WG was capable of reducing both the growth rate and spore germination up to 50% at 5 ppm concentration. However, a 140 fold increased dose was needed for effective control of *A. solani* under in vivo conditions. The reason for this may be that in the poisoned food technique fungicidal effect was evaluated in a controlled environment where only the pathogen was directly interacting with the fungicide, while under in vivo conditions several factors were involved such as fluctuations in environmental factors, a complex mixture of pathogens, dilution factors (e.g., rain, irrigation etc) and host resistance.

Spore germination and growth rate inhibition occurred after application of trifloxystrobin (one component of Nativo®75 WG). Trifloxystrobin is a broad-spectrum foliar fungicide that has high levels of activity against

many fungal pathogens within the Ascomycota, Basidiomycota, and Oomycota (Pesticide fact sheet, US Environmental Protection agency, September, 1999). Trifloxystrobin causes structural changes in the fungal mycelia (Taechowisan et al, 2005), abnormal growth of hyphae such as stunted, shortened and highly branched and bipolar or vesicular tip of swollen germ tubes (Kobayashi *et al.* 2005), swelling of hyphae with abnormal deposition of chitin, decrease in hyphal length, and changes in fungal metabolic activity (Yekutiel *et al.* 2004).

Trifloxystrobins are part of the larger group of QoI inhibitors, which inhibit mitochondrial respiration by influencing the function of the cytochrome bc1 complex (complex III), which is located in the inner mitochondrial membrane of fungi (Ziegler *et al.* 2003). Production of reactive oxygen species (ROS) from mitochondrial inter and inner membrane spaces is an unavoidable consequence of aerobic respiration (Chance *et al.* 1979). The mitochondrial electron transport system (ETS) is the major site of ROS production in non-photosynthesizing plant cells (Puntarulo *et al.* 1991; Halliwell & Gutteridge 2007). Depending on the mitochondrial respiratory states, a small portion of the consumable oxygen is partially reduced to generate ROS (Skulachev 1996; Turrens 1997; Møller 2001; Considine *et al.* 2003; Smith *et al.* 2004). The monoelectronic reduction of oxygen by ETS leads to the production of superoxide radicals ($O_2^-$) that can be dismutated by SOD, producing hydrogen peroxide ($H_2O_2$), and further decomposed by catalase and/or ascorbate-glutathione peroxidase cycles (Møller, 2001). An imbalance between the ROS production and antioxidant defenses can lead to an oxidative stress condition (Camacho-Pereira *et al.* 2009). In this present work, it is noteworthy that, after the fungicide treatment at $EC_{50}$, the activity of SOD, CAT and PO were highest on the 8$^{th}$ day following treatment. The gradual increase of superoxide radicals and hydrogen peroxide is clear evidence of a higher respiration rate. However, on the 12$^{th}$ day after treatment, the increase in SOD activity was higher than that of PO and CAT. Due to this imbalance, an oxidative stress condition appeared where the concentration of hydrogen peroxide increased. The undesirable accumulation of ROS causes oxidative damage of mitochondrial proteins and leads to the collapse of mitochondrial membrane potential (Qin *et al.* 2011) which leads to fungal death. Oxidative damage, and increases in the activity of SOD, CAT, PO and hydrogen peroxide content increase can be correlated with the decrease in total phenol concentration within plant cell treated with UV light (Agarwal 2007). Phenolics are the substrates for the peroxidase enzymes (Kar & Mishra 1976). In our present work, the decreased phenol content after fungicidal treatment has strengthened the above view in a fungal system.

A high carbohydrate status has been associated with elevated respiration rates in a number of studies (Moser *et al.* 1982). It is known that under conditions of increased respiration, the total carbohydrate content decreases as it is utilized by the glycolytic and Kreb's cycle pathways. An increase of total protein content in fungal tissue can be apparently correlated with the increased activity and concentration of different stress enzymes after fungicidal treatment in fungal tissue.

When conidia of a potential fungal pathogen land on plant surfaces, plant waxes may induce the production of extracellular esterases in germinating spores that digest hydrophobic esters in the wax layer (Berto *et al.* 1999). The esterases of pathogenic conidia, including cutinase, are also important factors for pathogen adhesion to the host plant. These enzymes allow germinating conidia to acquire nutrients from the leaf cuticle for successful infection (Jansson *et al.* 2003). In this work, the continuous decrease in esterase activity suggests the continuous loss of aggressiveness of the pathogen after fungicidal treatment.

Ergosterol is a major component of the fungal plasma membrane. In the presence of tebuconazole, (ergosterol biosynthesis inhibitor) formation of the plasma membrane is hampered. The ergosterol biosynthesis inhibition and decreased esterase activity are reflected in the inhibition of spore germination in our study.

From our work it is clearly evident that Nativo®75 WG is a broad spectrum fungi-toxic product which inhibits the growth of *A. solani*. The fungicide alters the oxidative balance and ergosterol biosynthesis of plant pathogenic *A. solani* infecting tomato. The increase in the oxidative stress-related enzymes up to 8 days after Nativo®75 WG treatment indicates that the pathogen is trying to establish itself in a fungicidal stress environment. The subsequent decrease from 8 to 12 days after treatment indicates fungal tissue death under fungicidal stress at the $EC_{50}$ dose.

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