USE OF TRICHODERMA SPP. TO IMPROVE PLANT PERFORMANCE UNDER ABIOTIC STRESSES

by Fatemeh Mastouri

USE OF *TRICHODERMA* SPP. TO IMPROVE PLANT PERFORMANCE UNDER ABIOTIC STRESSES

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This dissertation is mainly focused on the effects of *Trichoderma* spp. on plant performance under abiotic stresses. Chapter one examines the effects of *T. harzianum* on tomato seed germination and seedling growth under a spectrum of abiotic stresses that commonly happen during seed germination and adversely affect crop performance. Results of these experiments clearly demonstrate that the fungi enhance germination and plant performance under a variety of stresses. It is postulated that *Trichoderma* may enhance redox buffer capacity of treated plants and thereby enhance their tolerance to a range of abiotic stresses. This hypothesis was examined in chapter two of this dissertation. The expression of genes encoding antioxidants and their activity was shown to be improved by *Trichoderma* treatment. In parallel, the redox state of major antioxidants, glutathione and ascorbic acid were improved by *Trichoderma* treatment. The effects of such changes in the antioxidant capacity of plants, on photosynthetic efficiency of tomato plants under stress are examined in chapter three. Finally in chapter four our current understanding of plant-*trichoderma* interaction is summarized and is compared with that of other known beneficial fungi.

BIOGRAPHICAL SKETCH

Author of this dissertation was borne in Tehran, Iran. After receiving her high school diploma in empirical sciences, she attended College of Agriculture at University of Tehran and obtained a Bachelor of Science degree in Horticulture in 2002. she continued her studies at graduate level at Tarbiat Modarres University of Theran and received a Master of Science degree in Horticulture at 2005. to pursue her interest in the use of beneficial microbes in agriculture, she moved to Cornell University in 200, where she studied this subject under supervision of Drs. Gary E. Hraman and Thomas Björkman who are well-known for their ground-breaking studies of the topic.

This dissertation is dedicated to my favorite teacher, my mother who thought me the first lessons I have ever learnt; to my father who thought me with his hard work that there is no impossible in this world and to my beloved sister and brother who supported me in every step that I took in my life. It is also dedicated to my beloved friend Dr. Babak Kouchmeshky that inspired me to never stop seeking knowledge

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PREFACE

Trichoderma genus is an economically important genus within *Hypocreaceae* family (72, 94, 103, 174, 184). The fungi of this genus are ubiquitous and mainly reside in soil (72, 103, 183). Some species or individual strains within species of *Trichoderma* are associated with enhanced plant growth and productivity (22, 98, 107, 110, 201, 202, 250) and suppression of plant diseases (53, 64, 94, 98, 100, 103, 110, 183, 202, 234). Some species including *T. reesei* (21, 114, 221) and *T. harzianum* (102) have industrial applications. Some species or strains within species with negative economic or health impacts are also known (4, 138, 185). Due to the great economic impact of this genus, some species were among the first organisms to be fully sequenced (72). Currently eighty nine species of *Trichoderma* or *Hypocrea,* teleomorph of *Trichoderma*, are named and many more may emerge as molecular tools are being used to assist taxonomy of the genus (72, 184).

The main impact of *Trichoderma* genus on humans is through use in crop production (72, 94, 103, 183). Groundbreaking observations (7, 240) during early twentieth century suggested that *Trichoderma* species could parasitize other fungi and suppress diseases. Since then, about 17,000 scientific papers and over 100 patents are published only on the use of fungi of *Trichoderma* as biocontrol agents. The tremendous amount of information compiled over more than seven decades has uncovered various modes of action and benefits of this fungi on disease control and plant growth (23, 52, 53, 92, 94, 103, 118-120). In a pioneering review of current knowledge of *Trichoderma*-plant-pathogen interaction, Harman and associates suggested that the fungi establish a symbiotic relationship with host plants (103), which is in many cases mutualistic (100). Recent observations in commercial and academic trials started to shed light on a new mode of action of *Trichoderma* on plant growth (19, 33, 98, 148, 149, 202, 251); *Trichoderma* spp. induce tolerance to abiotic stresses in symbiotic plants. This thesis

was an attempt to better elucidate this phenomenon and expand our knowledge on this topic. In all of our studies tomato is used as the model organism, and extrapolating these results to other crops must be done with care. Also we have used a few highly selected strains of *Trichoderma,* and the results explained throught this dissertation, might not be applied when other strains are used.

In the first chapter, we will discuss the effects of *Trichoderma harzianum* on germination of tomato seeds under diverse abiotic stresses. Evidence will be presented that this fungi induces plant antioxidant defense mechanism. The second chapter focuses on the effects of this fungus on the antioxidant capacity of treated seedlings. The changes in expression and activity of antioxidant enzymes and the redox state of major non-enzymatic antioxidants in response to *Trichoderma*-treatment and water deficit will be addressed. The effects of these changes in plant tolerance to abiotic stresses and their importance for crop production will be discussed. The third chapter, which is an extension of chapter two, tests whether induction of antioxidant defense pathways, especially in chloroplast, may protect photosynthetic machinery under water deficit and salinity. The two stresses are chosen based on their worldwide impact on crop production. Changes in the rate of photosynthesis and its correlation with stomatal and non-stomatal limitations to photosynthesis during abiotic stresses will be discussed in this chapter. Fourth chapter summarizes our current knowledge of the fungal biocontrol agents and their interaction with host plants.

CHAPTER 1

Seed treatments with *Trichoderma harzianum* alleviates biotic, abiotic and physiological stresses in germinating seeds and seedlings

Abstract

Trichoderma spp. are endophytic plant symbionts that are widely used as seed treatments to control diseases and to enhance plant growth and yield. While some recent work has been published on their abilities to alleviate abiotic stresses, specific knowledge of mechanisms, abilities to control multiple plant stress factors and their effects on seeds and seedlings is lacking. We examined the effects of seed treatment with *T. harzianum* strain T22 on germination of seeds exposed to biotic stress (seed and seedling disease caused by *Pythium ultimum*) and abiotic stresses (osmotic, salinity, chilling, or heat stress). We also evaluated the ability of the beneficial fungus to overcome physiological stress (poor seed quality induced by seed aging). If seeds were not under any of the stresses noted above, T22 generally had little effect upon seedling performance. However, under stress treated seeds germinated consistently faster and more uniformly than untreated seeds whether the stress was osmotic, salt or suboptimal temperatures. The consistent response to varying stresses suggests a common mechanism through which the plant-fungal association enhances tolerance to a wide range of abiotic stresses as well as biotic stress. A common factor that negatively affects plants under these stress conditions is accumulation of toxic reactive oxygen species (ROS), therefore we tested the hypothesis that T22 reduced damages resulting from accumulation of ROS in stressed plants. Treatment of seeds reduced accumulation of lipid peroxides in seedlings under water deficit or in aged seeds. In addition, we showed the effect of exogenous application of an antioxidant,

glutathione, or application of T22, resulted in similar positive effect on seed germination under water deficit or in aged seeds. These evidences support the model that *Trichoderma harzianum* strain T22 increases seedling vigor and ameliorates stress by inducing plants physiological protection against oxidative damage.

Introduction

Trichoderma spp. have been known as biocontrol agents for the control of plant diseases for decades (103, 240). However, we now understand that biocontrol is, in many cases, not related to their abilities to produce antibiotics, establish parasitic interactions, or otherwise directly affect pathogens (103, 119, 120, 247). Instead, it is now clear that in most cases the beneficial fungi induce systemic resistance that is mediated by alterations in plant gene expression (6, 103, 110, 201, 202).

There also are reports of enhanced plant growth as a result of the association of *Trichoderma* strains with plants, but the effect, as with other plant growth promoting microbes (83), are greater when plants are under suboptimal conditions or biotic, abiotic or physiological stresses (19, 98, 103, 149, 202, 251). Several recent reports indicate that the fungi enhance tolerance to abiotic stresses during plant growth (19, 33, 98, 251), in part because of improved root growth or improvement in water holding capacity of plants (19, 98), or enhancement in potassium uptake (251), while in absence of stress plant growth may (19) or may not (251) be enhanced. Although molecular studies indicate greater expression of families of genes involved in protection against abiotic stresses or oxidative damage (5, 6, 22, 24, 201), no experimental evidence has been presented to correlate enhanced tolerance of plants colonized with these fungi to these changes in molecular level.

These fungi are frequently applied as seed treatments, where they may improve plant

stands and induce long-term improvements in plant quality (98, 100). Seed treatments therefore, can induce both short-term and long-term improvements in seed and subsequent plant performance, but little is known about the early seed-*Trichoderma* interactions. These interactions are important because (a) they can provide insights into long-term plant performance, and (b) seed-*Trichoderma* interactions, if properly characterized and quantified, can provide powerful and rapid systems to examine mechanisms and physiological processes of the plant-*Trichoderma* interactions. We created a series of testable hypotheses in this study to better understand the nature of effect of *Trichoderma* on tolerance to abiotic stresses, as follows:

1. Seeds respond positively to treatment with *T. harzianum* when exposed to physiological, biotic or abiotic stresses, but the beneficial fungus has little or no effect on seeds not exposed to these stresses.
2. Treatment of seeds with *T. harzianum* ameliorates a wide variety of biotic, abiotic and physiological stresses to seeds and seedlings. To the best of our knowledge, there has been no other systemic study on the abilities of the fungus to improve performance of seedling or growing plant across a variety of stressful conditions. Instead, other few studies have focused on only one stress at a time and due to the differences in the experimental conditions, including differences in plant species or fungal species, comparisons are hard to make.
3. Seeds respond to *T. harzianum* very early during germination, i.e., before radicle protrusion. As mentioned, the prevailing hypotheses in this area revolve around the enhanced root growth or plant enhanced water holding capacity, but if seed germination under stress is enhanced, an alternative explanation is required.

The goals of this study were (1) to evaluate the ability of *T. harzianum* to improve germination and seedling performance in good quality seeds in the absence of stress,

and with stresses imposed by seed aging (physiological stress) or unfavorable osmotic, salinity, or temperature conditions as well as biotic stress (attack by the seed and seedling pathogen *Pythium ultimum*) and (3) to determine whether amelioration of toxic effects of reactive oxygen species (ROS) may be involved in beneficial effects of the fungus on seeds under stresses.

Materials and Methods

Plant material and seed treatment

Seeds of tomato (*Lycopersicum esculentum* L.) cultivar 'Jubilee' (Harris seeds, Rochester, NY) were used in this study. To reduce seed quality, seeds were artificially aged (39, 87). Briefly, seeds equilibrated to 70% relative humidity were incubated for various periods (0, 5, 10, 15, 20 days) at 38˚C in sealed aluminum pouches.

Trichoderma harzianum Rifai Strain T-22 (ATCC # 20847) was used for this study. This strain is rhizosphere competent and is capable of inducing systemic resistance and stimulating plant growth (33, 100, 110, 201). It induces systemic changes in the transcriptome and proteome (109, 201, 203, 247), even though it colonizes only few layers of root cortex and epidermis (247). To prepare seed treatment inoculants, conidia coated onto cellulose and encapsulated with tapioca dextran (Crystal-Tex, National Starch, NJ) were suspended in sterile type I water. Seeds were treated with the conidial suspension at rate of 20 $\mu l.g^{-1}$ to deposit 2×10^7 cfu per gram of seeds. Control seeds were treated with an equal amount of type I sterilized water.

Seed germination test and seedling growth

In vitro condition: Seeds were germinated in clear plastic boxes (12×12×5 cm) with

lids. One hundred seeds of each treatment were placed on two layers of blotter paper moistened with 16ml of sterile type I water for each replicate. Each experiment included four replicates and was repeated at least twice. Seeds were incubated at 25±1°C and 16 h of light. Germination was measured every 12 h and seeds counted as germinated when the radicle protruded through the seed coat. The percentage of normal seedlings was measured after 14 days according to standard germination testing (13). Time to germination of fifty percent of seeds, hereafter, T_{50}, an indicator of average speed of germination (87), and a sensitive measure of vigor was measured.

To quantify the effect of T22 treatment on plant growth and fitness *in vitro*, unaged or rapidly aged seeds treated or untreated with T22 were planted in sterilized Magenta boxes half-filled with 0.8% water agar. Twenty five seeds of each treatment were planted in each box and were incubated at 25±1°C with 16 h of light of approximately 180µmol.$m^{-2}s^{-1}$. Seedlings were harvested 14 days after planting (DAP) and radicle and hypocotyl fresh weight (FW) and length were measured. The radicles or hypocotyls of, normal seedlings of each experimental unit were pooled and dried at 65°C for 48 hours. Average DW was calculated as the DW of the pool of radicles or hypocotyls in each replicate divided by the number of normal seedlings in that unit.

Germination in *Pythium*-infested soil: To compare the effect of *T. harzianum* on germination in presence or absence of diseases, seedling emergence was also studied in soil infested with *Pythium ultimum* strain P_4. Sporgania were produced according to Taylor et al. (88, 218) (111) and the level of inoculant was adjusted to the level that caused 25% mortality in beans. Small transparent boxes measuring 15×15× 5 cm, were filled with either autoclaved Arkport sandy loam (as a control) or *Pythium*-infested sandy loam (hereafter infested soil). Twenty fresh seeds of 'Jubilee' tomato, either treated with T22 or not treated were planted in each box as a replicate. Each

treatment was replicated eight times. Boxes were incubated in a growth chamber with 16 h of light and 25±1°C. Seedling emergence was measured every 24 hours and plants were seedlings that died from damping off were counted. Plants were harvested at 14 DAP, when FW and length of radicles and hypocotyls were determined. Average DWs for each experimental unit (an individual box) was determined.

Effect of *Trichoderma* treatment on germination under abiotic stresses

Water deficit: Seeds of 'Jubilee' tomato, treated with T22 or untreated were planted in 0.8% water agar (w/v Difco agar) plates (control). Polyethylene glycol (PEG) (MW 8000; Sigma, St Louis, MO) was added to lower water potential. Water potentials used are hereafter denoted as P1 (-0.1 MPa), P2 (-0.2 MPa), or P3 (-0.3MPa) (226). Five replications of 100 seeds were planted and were incubated at 25±1 ºC with 16 hours of light and germination was measured every 24 hours for up to 14 days. The percentage of normal seedlings was determined after 14 days according to the AOSA guideline (13). Radicle and hypocotyl FW and length were determined and then the average DW of radicles and hypocotyls of each experimental unit (100 seeds) was determined.

Salinity stress: Tomato seeds, treated with T22 or untreated, were planted in 0.8% water agar media with 0, 50, 100, or 150 mM NaCl (respectively control, S1, S2, or S3). Seed germination tests were carried out as previous section and T_{50}, percentage of normal seedlings, radicle and hypocotyl FW, and average DW were determined.

Chilling or heat stress: To expose seeds to chilling or heat stress during germination, unaged seeds of tomato cultivar 'Jubilee', treated with T22 or untreated, were planted on blotter paper as described above, but after 24 h of the start of seed imbibition, seeds were transferred to either 10 ± 0.2 ºC (chilling stress) or 35 ± 0.5 ºC (heat stress) for 24 or 72 h. After exposure to these temperatures, seeds were returned to the

germination chambers. Germination was measured every 12 h and each treatment was allowed to germinate and grow for up to 14 days in a germination chamber. Seedling growth, radicle and hypocotyl FW and average DW, were measured after 14 days.

Measurement of lipid peroxides

Lipid peroxidation is commonly used as an indicator of oxidative damage. We measured lipid peroxides in tomato seedlings grown in the presence or absence of the water deficit or seed aging. The two stresses were chosen because they represent independent sources of stress to the germinating seeds. To obtain samples of comparable physiological age, all seedlings with radicles measuring 5mm or longer were harvested, when approximately 50% germination was achieved. Total lipids was extracted following Hara and Radin (91) with modifications. Briefly, 0.5 g of ground sample was mixed with 7 ml of hexane/isopropanol (3:2) and was centrifuged for 10 minutes at 5,000 rpm. The supernatant was collected and pellet was centrifuged again with an additional 2 ml of extraction media. Supernatant from two steps was combined and lipid peroxides were determined using colorimetric assay with the PeroxiDetect^{TM} kit (Sigma Aldrich, MO) according to manufacture's direction.

Exogenous application of glutathione (GSH)

To determine whether enhancing antioxidant buffer capacity of plants is sufficient to enhance germination in a similar manner as T22-treatment, GSH was exogenously applied to germinating seeds**.** GSH was added to germination media at a final concentration of 3mM according to (37). Treatments included control, lowered water potential media of P1 (-0.1 MPa) ±T22 or ±GSH and seeds aged for 20 days ±T22 or ±GSH. Seeds were incubated at 25±1ºC with 16 hours of light. Germination of seeds was monitored and final germination percentage was determined 14 days later.

Statistical analysis

A factorial design was used for all of the experiments. Factors tested in each experiment were the main effects, T22 (+/-) and source of stress, i.e. aging, osmotic stress, salinity, chilling or heat, and interaction between T22 and stress. Each experiment consisted of four replicates and was repeated. Data were analyzed by the analysis of variance (ANOVA) using JMP 7 for Windows (SAS®) statistical package. Means were compared using a t-test at α=0.05, to determine whether the impose level of stress, T22 or their interaction had significantly affected the speed of germination, percentage of germination, root or shoot fresh or dry weight. To compare the distribution of seedling sizes, the Shapiro-Wilk test of normality was used from JMP7.

To determine the T_{50} values, a non-linear logistic model was fitted to germination data of each repetition. Several models were tested and the goodness of fit was compared and a logistic model with three predictors as follows was selected to determine T_{50}:

$$g = \frac{\theta_1}{1 + \theta_2 + e^{\theta_3 \times t}}$$

Where g is germination at each time, t, and θ_1, θ_2, θ_3 are parameters describing the germination curve.

Results

Effects of T22 treatment on germination and early seedling growth

The speed of germination and germination percentage were measured. Germination percentage is commonly measured as an indicator of seed viability, but it is insufficiently sensitive to detect small changes in seed quality that affect performance of seeds under field conditions. However, speed of germination is more sensitive and

can reflect even small changes in seed quality that affect field performance (87). T22 treatment increased the speed of germination (lower T_{50} values) in unaged or aged seeds of 'Jubilee' tomato (P=0.0006). The effect of this treatment was greater in aged seeds than in fresh seeds, but the interaction between the two factors of aging and T22 treatment was not statistically significant. The percentage of germination as increased in response to this treatment in aged seeds, but not in unaged seeds (P=0.0023).

Radicle and hypocotyl length (growth) and FW of seedlings (biomass accumulation) produced from unaged seeds of 'Jubilee' tomato two weeks after planting in aseptic culture were not affected by T22 treatment (P=0.3564) (Table 1.1).

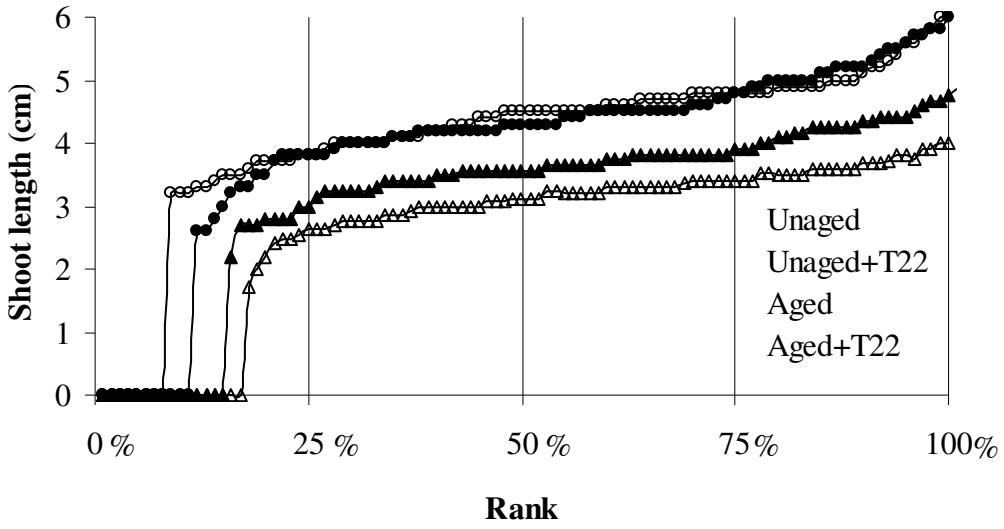

Figure 1. 1. Distribution of shoot length as affected by rapid aging and T22 treatment.

Seedling growth was compromised due to rapid aging (P=0.0001), but T22 treatment partially restored growth (P<0.0001, using the Shapiro-Wilk's test) (Fig. 1.1). Seedlings produced from T22-treated aged seeds had greater growth (hypocotyl and radicle length) and biomass accumulation (shoot and radicle FW & DW) (Table 1.2) than did seedlings from untreated seeds.

Seedling emergence in soil infested with *Pythium ultimum*

Neither seed treatment nor *Pythium* infestation of soil affected speed of germination or percentage of seedlings emerging. However, 15% of seedlings from untreated seeds were lost due to post-emergence damping off, while there was no seedling mortality in seedlings produced from T22-treated seeds. *Pythium* infestation of soil reduced root dry mass significantly (*P<0.0001,* 1.14±0.17 g/plant in the control and 0.58±0.17g in *Pythium*-infested soil), but shoot dry mass was not affected. The mass of roots from T22-treated seeds was 46% higher than seedlings without the beneficial fungus.

Table 1. 1. The effect of T22 on germination percentage and speed of germination (T_{50}) of tomato seeds

Treatment	Germination %		T_{50} (h)	
	-T22	+T22	-T22	+T22
A0*	94.5 ± 0.73 a[xy]	94.5 ± 0.65 a	59.7 ± 1.97 ab	56.6 ± 0.89 a
A10	90.3 ± 0.76 a	91.1 ± 0.85 a	64.8 ± 2.95 b	57.6 ± 1.03 a
A20	86.6 ± 0.88 b	89.3 ± 0.88 a	70.0 ± 1.85 c	63.1 ± 1.19 b

***** A0, A10 or A20 respectively stand for unaged seeds, or seeds rapidly aged for 10 or 20 days
[x] Each number is average of 8 replications in two experiments ± standard error of the mean (SEM**)**
[y] Averages followed by the same letter are not statistically different according to the student's t least significant difference test

Table 1. 2. The effect of T22 on growth of tomato seedlings germinated in aseptic media and were harvested after 14 days of growth

	Shoot length (cm)		Root length (cm)		Shoot FW (mg)		Root FW (mg)	
	-T22	+T22	-T22	+T22	-T22	+T22	-T22	+T22
A0	4.45 a [x]	4.41 a	8.22 a	7.77 ab	31.62a	31.45 a	10.38 a	10.05 a
A10	3.91 c [y]	4.19 b	7.10 bc	7.50 b	28.16b	30.01ab	9.68 b	9.93 a
A20	3.21 d	3.74 c	5.95 d	6.93 c	22.97c	27.42 b	8.69 d	9.12 c

[x] Each number is average of 8 replicates in two experiments
[y] Averages followed by the same letter are not statistically different according to the student's t least significant difference test

Trichoderma treatment enhanced germination and growth under abiotic stresses

Water deficit: to study the effect of seed treatment with T22 on germination of seeds under water deficit, seeds were planted in water agar plates with lowered water potential. Germination of seeds was slowed by reduction in the water potential of the germination media and final germination, measured 14 days after the start of experiment, was severely affected by water deficit especially at -0.2 and -0.3MPa (Fig. 1.2 & 1.3). However, seed treatment with T22 enhanced speed of germination ($P<0.0001$) and seeds treated with T22 germinated faster and more uniformly compared to untreated seeds at all osmotic stress levels (Fig. 1.2). Seedling growth was also improved by T22 treatment (Table 1.3).

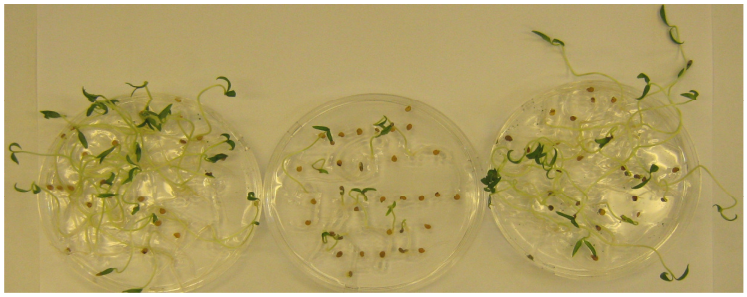

Figure 1. 2. T22 improved germination of tomato seeds under water deficit. From left to right control, media with -0.2 MPa, T22-treared seeds in media with -0.2 MPa.

Salinity: salinity significantly reduced speed and percentage of germination (Fig. 1.4). T22 treatment enhanced speed of germination (lower T_{50} values) at all elevated salinity levels and percentages of germination in S2 (Fig. 1.4). It also partially restored the vigor of seedlings under salinity (Table 1.3).

Chilling and heat stress: Less than 5% of seeds incubated at 35 ºC for three days germinated during this treatment and no germination was observed in seeds imbibed for 1-3 days at 10 ºC or 1 day at 35 ºC. Both chilling and heat stresses reduced germination speed compared to control condition (Fig. 1.5 *a,b*). Longer exposure to low temperature (3 days reduce germination speed more to one day exposure, but

there was a significant reduction in vigor when seeds were exposed to heat for three days compared to one day (Fig. 1.5 *a,b*). T22 enhanced speed of seed germination (P=0.0006) and treated seeds germinated faster than untreated seeds under all combinations of heating or chilling stress periods. There was not a significant interaction between T22 application and chilling or heat stresses. Although T22-treated seeds germinated significantly faster than untreated seeds, the final germination percentage was not significantly different.

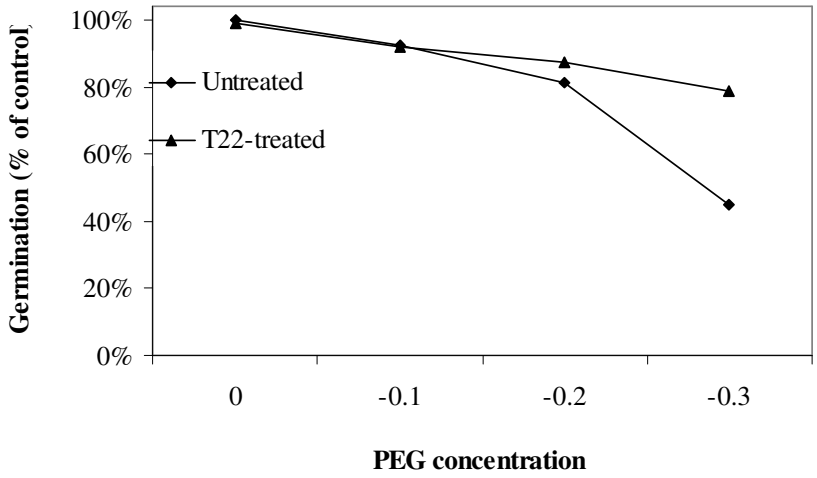

Figure 1. 3. The effect of T22 and osmotic stress on germination of tomato seeds; relative germination% in PEG solutions with -0.1, -0.2, and -0.3MPa are shown.

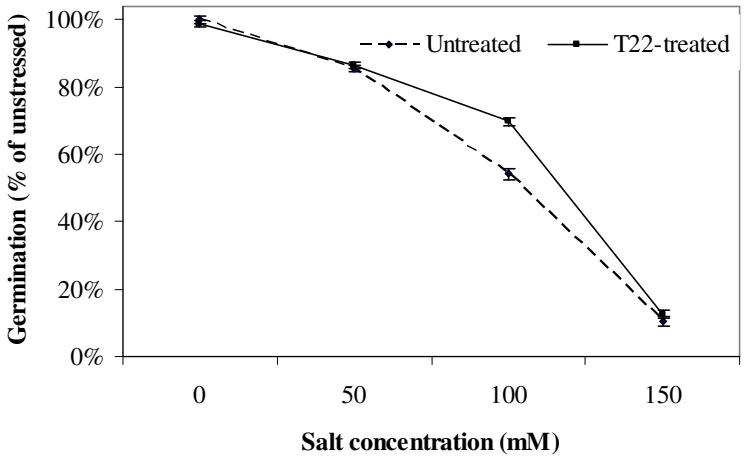

Figure 1. 4. The effect of T22 and salinity on germination% of tomato seeds; relative germination% in media with 50, 100, and 150mM NaCl are shown.

Table 1. 3. Effect of T22, water deficit or salinity on seedling growth

		Shoot length (cm)		Root length (cm)	
Source of stress	Level	-T22	+T22	-T22	+T22
Control		4.29± 0.16 a[y,z]	4.12±0.10 a	7.92±0.20 a	7.58±0.16 a
Osmotic stress	P1*	3.59±0.17 abc	3.89±0.11 ab	7.10±0.22 ab	7.49±0.17 a
	P2	2.91±0.13 c	3.65±0.21 ab	6.50±0.19 c	7.02± 0.12 ab
	P3	2.04±0.24 d	3.09±0.18 bc	5.17±0.26 d	6.38±0.17 c
Salinity	S1[x]	3.21±0.29 bc	3.62± 0.15 ab	6.95±0.13 ab	7.54±0.11 a
	S2	2.05±0.27 d	2.99± 0.23 c	5.26±0.24 d	6.95±0.21 ab

* P1, P2, P3 respectively denote media with -0.1, -0.2, -0.3 MPa water potential
[x] S1 and S2 denote media containing 50 and 100mM NaCl
y Each number is average of 8 replications in two experiments ±SEM
z Averages followed by the same letter are not statistically different according to the student's t least significant difference test

T22 treatment reduces lipid peroxidation: Total lipid peroxides of seedlings produced from aged seeds or those germinated under lowered water potentials was compared between T22-treated or control seedlings. To obtain samples with similar physiological age, samples were harvested when approximately 50% of seeds were germinated. Harvest times were as followed: control, T22 treated fresh or 20 day aged seeds, and -0.1 MPa+ T22: 5 DAP, untreated seeds aged for 20 days: 7 DAP, -0.1 MPa and -0.2 MPa +T22: 8 DAP, and -0.2MPa: 10 DAP. T22 treatment and osmotic stress significantly affected lipid peroxide levels (respectively $P=0.0350$, $P<0.0001$), but the effect of seed aging was not significant ($P= 0.2391$). Lipid peroxide content increased 8-fold in plants grown at -0.2 MPa compared with control seedlings. Lipid peroxide content of T22-treated seeds also increased with the reduction in water potential, but at -0.2 MPa, significantly lower concentrations of organic peroxides were detected in T22-treated seedlings compared with the control (Table 1.4).

Exogenous glutathione application: Exogenous application of 3mM glutathione

(GSH) counteracted the negative effect of osmotic stress and seed aging on germination of seeds under lowered water potential. T22 treatment and GSH treatment increased seed germination by similar amounts (Fig. 6). Exogenous application of GSH gave a numerical increase in germination percentage germination of T22 treated seeds but the level was not significant (data not shown).

Table 1. 4. Lipid peroxide content (μmol.gr^{-1} fw) of tomato seedlings untreated or treated with T22 under water deficiy (control, P1, and P2).

Osmotic potential	Lipid peroxide (μmol.gr^{-1})	
	-T22	+T22
Control	1.1±0.7 c^{x,y}	0.8±0.9 c
P1 (-0.1MPa)	3.1±1.0 b	2.2±1.1 bc
P2 (-0.2 MPa)	8.5±2.4 a	4.1±1.2 b

[x] Numbers are average of three replications ± SEM
[y] Averages followed by the same letter are not statistically different according to the student's t least significant difference test.

Discussion

A single treatment of seeds or plants that would simultaneously confer resistance to biotic stresses (disease) and abiotic stresses would be of importance to agricultural plant production. This report demonstrates that seed treatments with *T. harzianum* are capable of alleviating abiotic and physiological stresses in seeds and seedlings. *Trichoderma* spp. are among the most widely used organisms for biological disease control and for plant growth promotion (103, 110). This report also shows that they induce tolerance to abiotic stresses and indicate that there is a great opportunity to improve plant agriculture by fully utilizing their capabilities.

A few recent reports demonstrated that these fungi alleviate abiotic stresses. Field data indicate that they may confer tolerance to drought stress at least in part through promotion of deeper root penetration into the soil profile (98). In a recent report, *T.*

hamatum increased tolerance of cocoa plants to water deficit through increasing root growth that provided greater water resources to treated plants and delayed onset of water deficit in these plants (19). In another report (251), squash plants treated with *T.*

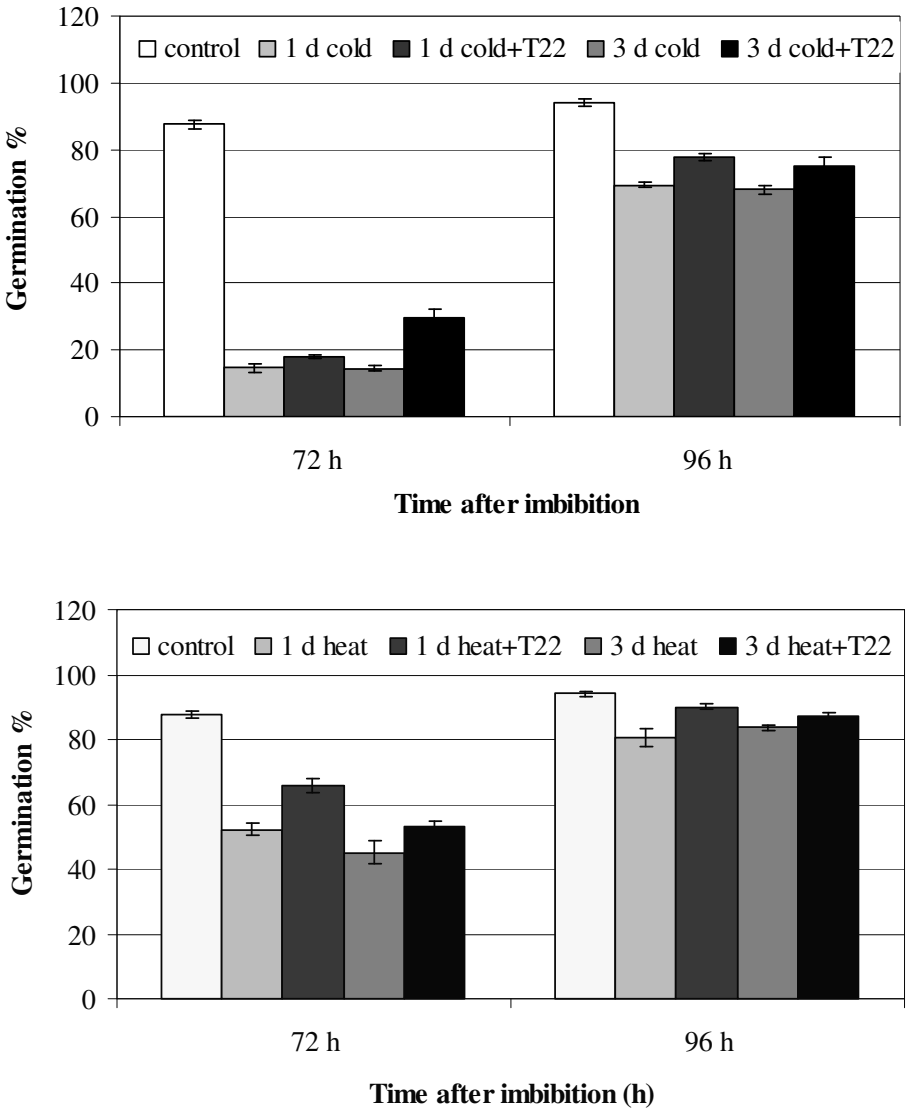

Figure 1. 5. The effect of T22 on germination of 'Jubilee' tomato seeds exposed to chilling or heat stress; seeds were imbibed for 24 h at 25±1ºC and then exposed to either 10 ºC for either 1 or 3 days (top), or 35ºC for 1 or 3 days (bottom).

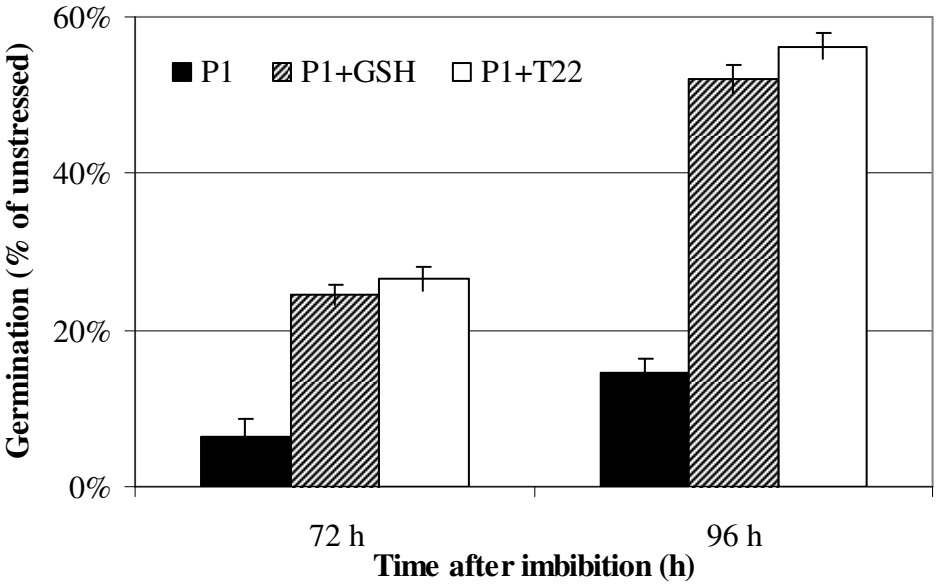

Figure 1. 6. Germination of tomato seeds as affected by water deficit, GSH or T22. Percentages of germination at 72 and 96 hours are shown here. Each value is average of two experiments each with fours replicates±SEM.

harzianum strain T22 or other beneficial microorganisms were more tolerant of salinity than untreated ones. These data, along with the frequent observation that the greatest advantage to *Trichoderma* treatments of plants occur when they are under stress, gives credence to the concept that these beneficial fungi ameliorate both biotic and abiotic plant stresses.

However, there are almost no reports of the abilities of *Trichoderma* spp. to alleviate stresses that occur in the seed and early in germination. An exception was the report by Björkman (33) that a seed treatment with *T. harzianum* could confer advantages to maize seeds with poor vigor caused by genetic manipulations (early varieties of sweet corn with the *sh* gene), increasing both vigor and germination. The present study examined effects of T22 treatment of tomato seeds on several physiological and abiotic stresses during imbibition and germination. All stresses examined were alleviated by T22 treatment, including water deficit and salinity, changes in

temperature and loss of seed quality due to storage conditions that give rise to physiological intrinsic stress. This is of substantial importance to plant agriculture since yields and productivity of plants usually require high and uniform seed germination and strong seedling vigor.

Despite of significance difference between these stresses, signaling pathways and cellular responses to them share common features (1, 19, 38, 54, 78, 79, 81, 129, 155, 166, 181, 182). Therefore, eventhough a wide range of stresses were alleviated by T22 treatment, there may be similar mechanisms induced by the fungus resulting in amelioration of all of the stresses applied. One such common mechanism could be the control of damage caused by excess reactive oxygen species (ROS). These molecules that play crucial signaling role during abiotic stresses at higher concentration cause cellular and molecular damages (151). H_2O_2 is the most common ROS, which is either reduced to water during the ascorbate-glutathione cycle, is converted to water and oxygen by catalase enzyme, or is used as a substrate of peroxidase enzymes (78, 79, 161). However, in presence of metal ions, H_2O_2 is converted to hydroxyl radicals, which starts chain reactions leading to the peroxidation of membrane lipids (18) and damage to other macromolecules. Thus, measurement of lipid peroxide content serves as a reliable indicator of oxidative damage (151, 163) during abiotic stresses. We found an increase in level of lipid peroxide content in young seedlings germinated under water deficit, but T22-treated seedlings had significantly less lipid peroxide. Seed aging results in lipid preoxidation (108), but in this study, while numerical increases in peroxide levels were detected, and T22 reduced these, neither effect was statistically significant. Exogenous application of GSH was sufficient to enhance germination of seeds under water deficit, an effect similar to that of T22 treatment. GSH can directly react with a range of ROS (162). In addition, it is involved in

glutathione-ascorbate cycle during which H_2O_2 is reduced to water (161) and induces expression of Cu/Zn superoxide dismutase (115), an important ROS scavenging enzyme. These two lines of evidence support the model that *Trichoderma harzianum* strain T22 increases seedling vigor and ameliorates stress by inducing plants physiological protection against oxidative damage. An increase in the level of several families of protective proteins, including glutathione-dependent enzymes such as glutathione reductase, glutathione S-transferase (GST), and superoxide dismutase in *Trichoderma*-treated maize and other plants has been reported (5, 6, 20, 24, 201). The mechanisms whereby *Trichoderma* induce such changes are not known, but enhanced ROS level could act as a signal to regulate expression of some of the related genes. A transient increase in intracellular ROS has been detected 5-10 minutes after treating soybean cell culture with culture filtrate of *Trichoderma atroviride* (159). Such signals along with Ca^{++} signaling (159) can induce plant ROS scavenging mechanisms (151) resulting in elevated protection against the oxidative damage.

A question that may arise is whether fungi can tolerate and proliferate in conditions tested in this work. It is known that unlike many naturally occurring and selected strains of *Trichoderma* (104, 242), T22 is resistant to conditions resulting in oxidative damage such as application of fungicides that act by inducing lipid peroxidation (104) or application of paraquat at concentrations as high as 50 ppm (Mastouri and Harman, unpublished data). In addition, fungal biomass, sporulation or spore germination is not affected in media with water potentials as low as -2.0 MPa (127). Therefore, it is likely that the fungi can establish in such suboptimal conditions. However, these results may not be extended to other selected or natural strains without further studies.

Similar enhancement in tolerance to abiotic stresses has been observed as a result of association of other beneficial microorganisms with plants. Among effective agents,

plant growth promoting rhizobacteria (PGPR) and *Piriformaspora indica* mycorrhizal fungi could be noted to induce similar benefits. Other free-living rhizosphere competent fungi such as nonpathogenic Fusaria and binucleate *Rhizoctonia* (*Ceratorhiza* spp.) probably also belong in this list. All of these organisms directly colonize plants internally (27, 46, 85, 92, 126, 128, 135, 192, 230, 237, 247, 254). Many of them exhibit the same ranges of activity as T22, as recently summarized (94, 202). These results suggest that *Trichoderma* and some other endophytic microbes may alleviate a range of biotic, abiotic and even physiological stresses. All these benefits together with increasing nitrogen use efficiency (107, 197) indicate substantial commercial potential beyond current uses of these organisms.

CHAPTER 2

Trichoderma harzianum alters gene expression in tomato seedlings to enhance antioxidant defense systems and tolerance to abiotic stresses

Abstract

Some strains of *Trichoderma* spp. are plant symbionts that enhance resistance to biotic and abiotic stresses. They colonize roots and change plant gene expression. The effects of *Trichoderma harzianum* Rifai strain T22, a commercial strain of *Trichoderma,* on antioxidant defense of colonized seedlings and their response to water deficit was investigated. The pool size and redox state of glutathione and ascorbate, and the activity of antioxidant enzymes of glutathione-ascorbate cycle in response to T22 and water deficit were measured. The effect of T22 on expression of genes encoding enzymes of glutathione-ascorbate cycle, three superoxide dismutases, and two catalases were determined. To better understand the elements driving expression of these genes, prompters of 36 *Arabidopsis thaliana* genes encoding antioxidant enzymes and proteins catalyzing limiting steps of glutathione and ascorbate biosynthesis were analyzed. T22 treatment improved seedling growth and redox state of glutathione and ascorbate compared to control plants. Water deficit, on the other hand reduced concentration and the ratio of reduced:oxidized form of both molecules. Activity of glutathione-ascorbate cycle enzymes increased in response to T22. Similarly, expression of several genes encoding isoforms of glutathione-ascorbate cycle enzymes and chloroplast Fe and Cu/Zn superoxide dismutase was increased. This data suggest that T22 enhancement of tolerance to water deficit tightly correlates with enhancement in the glutathione-ascorbate cycle, a mechanism that may also improve tolerance to other biotic and abiotic stresses.

Introduction

Fungi in the genus *Trichoderma* and products based on the efficient strains thereof are commonly used to enhance yield and reduce plant diseases. *Trichoderma harzianum* strain T-22 (ATCC # 20847), hereafter T22, was produced by protoplasm fusion between *T. harzianum* T-95 and T-12 (99), and has been used both commercially and as model organism to study *Trichoderma* interactions with plants and pathogens (43, 94, 103, 107, 149, 201, 202, 231). This strain colonizes roots of diverse host plants (209) and causes systemic changes in host gene expression associated with induced systemic resistance (109, 201) and other beneficial effects (201).

Among the beneficial outcomes of *Trichoderma*-plant interaction, enhancement of plant resistance to abiotic stresses (19, 33, 149, 202, 251) has recently attracted more attention, as understanding the nature of this phenomenon may provide potentially useful tools to enhance crop production. In a recent study, we found that under stress, T22 improves germination of tomato whether the stress was water deficit, salinity, suboptimal temperatures or reduced seed quality (Mastouri*, et al.*, unpublished). The enhancement in germination and growth by T22 under conditions known to damage plant growth through enhanced production of damaging levels of ROS (14, 151) suggested that these changes might have been at least partially mediated by enhanced redox buffering capacity of the colonized plants. Under water deficit, lipid peroxide content of colonized seedlings was smaller than control (Mastouri, *et al.*, unpublished). Lipid peroxidation is commonly associated with oxidative damage (186) and occurs when the level of reactive oxygen species (ROS) exceed capacity of antioxidant defense system. ROS are partially reduced or activated derivatives of oxygen produced as byproduct of normal metabolism. Reaction center of PSI and PSII in chloroplast, mitochondria, and peroxisomes are major site of ROS production (14).

In normal growing conditions, level of ROS production is under tight control through a highly redundant network of enzymatic and non-enzymatic antioxidants (150, 151, 175) and as a result changes in ROS level may act as a signal to activate a host of defense mechanisms (151). Glutathione (GSH) and ascorbic acid (ASA) are the major non-enzymatic antioxidants responsible for maintaining cellular redox homeostasis through reduction of H_2O_2 to water in glutathione-ascorbate cycle (161). H_2O_2 reduction is catalyzed by ascorbate peroxidase (APX; EC 1.11.1.11), using reduced ascorbic acid (ASA) as electron donor. Oxidized ASA or monodehydroacrobate (MDHA) is either reduced to ASA in a reaction catalyzed by monodehydroascorbate reductase (MDHAR; EC 1.6.5.4), using NADH as electron donor, or is spontaneously converted to dehydroascorbate (DHA). Regeneration of ASA from DHA requires GSH as electron donor in a reaction catalyzed by dehydroascorbate reductase (DHAR; EC 1.8.5.1). Glutathione reductase (GR; EC 1.8.1.7) catalyzes reduction of oxidized glutathione or glutathione disulfide (GSSG), using NADPH as electron donor. Both glutathione and ascorbic acid are predominantly maintained in reduced form, but their redox state (percentage of reduced form), total pool size (oxidized + reduced form), and activity of enzyme involved in their regeneration (GR, MDHAR, and DHAR) changes in response to stresses and other stimuli (77, 78, 145, 161, 166). Enhanced activity of glutathione-ascorbate cycle enzymes, especially APX, GR, and MDHAR is correlated with enhanced tolerance to abiotic stresses (27, 152, 153, 211) and transgenic plants overexpressing or deficient in antioxidant enzymes provide definitive evidence about the role of antioxidants in defense against abiotic stresses (8, 80, 86). Recent work also correlates the enhanced antioxidant capacity of plants colonized with diverse endophytes to the increased tolerance to abiotic stresses (27, 186, 237).

The main goal of this study was to investigate the ability of T22 to modulate

antioxidant defense in tomato seedlings under water deficit. Using the model system previously reported (Mastouri, *et al.,* unpublished), we investigated (a) whether T22 treatment mediated an increase in total glutathione and/or ascorbate in colonized plants with or without water deficit, (b) whether the redox state of the molecules were enhanced, and (c) whether it mediated changes in the activity of glutathione-ascorbate cycle enzymes, namely APX, MDHAR, DHAR, and GR. Also, we determined if the changes in antioxidant defense were localized to roots or, whether any systemic changes occurred. Results that will be described here showed that the plant-*Trichoderma* association was not related to increase in total glutathione or ascorbate content. However, the redox state of both molecules was enhanced especially under stress, parallel to the enhancement in the activity of all four enzymes involved in glutathione-ascorbate cycle. Using quantitative real time-polymerase chain reaction (qRT-PCR) relative expression of genes encoding enzymes of glutathione-ascorbate cycle, two catalases (CAT) and three superoxide dismutases (SOD) in response to T22 was studied. Promoters of the genes encoding antioxidant enzymes and enzymes catalyzing the rate-limiting steps of GSH and ASA biosynthesis in *Arabidopsis thaliana* were analyzed to see whether DNA elements present in promoter regions of these genes may suggest how such genes are regulated

Materials and Methods

Plant material and seed treatment

Seeds of tomato (*Lycopersicum esculentum* L.) cultivar 'Jubilee' (Harris seeds, Rochester, NY) were used in this study. Conidia of T22 coated onto cellulose and encapsulated with tapioca dextran (Crystal-Tex, Bridgewater, NJ) were suspended in sterile type I water. Seeds were treated with 20 $\mu l.g^{-1}$ of the conidial suspension to

deposit 2×10^7 colony forming units per gram of seeds. Control seeds were treated with an equal amount of type I sterilized water.

Murashige and Skoog (MS) media in 0.25% phytagel (Sigma-Aldrich, MO) was used as germination and seedling growth medium. For stress experiments, PEG 8000 (Sigma-Aldrich, MO) was added after autoclaving media at 13.8 and 19.6% final concentration (-0.2 and -0.4 MPa respectively) to reduce water potential. Paraquat (Syngenta, DE) was added to MS media at a final concentration of 20μM after autoclaving. Seeds were incubated at 25±1°C and 16 h of light. Germination percentages were measured after 14 days. Chlorophyll content of hypocotyl of normal seedlings was determined with a pre-calibrated SPAD-meter (Minolta, US). Hypocotyl and radicle fresh weights were determined and then all hypocotyls and radicles in each replicate were pooled and dried at 65°C for 48 hours. The dry weight of each pool was then divided by the number of seedlings to determine average dry weight for hypocotyls and radicles. To determine enzyme activity and concentration of GSH and ASA seedlings were harvested 3 and 10 days after fifty percent of seeds germinated and were immediately immersed in liquid nitrogen. Enzyme activity was determined in whole seedling, but GSH and ASA concentrations were determined in radicles and hypocotyls separately. To analyze gene expression in response to T22, 20 seedlings treated with T22 or untreated from three independent studies were harvested 3 days after fifty percent of seeds germinated and were immersed in liquid nitrogen. Samples were ground in pre-chilled mortar and pestle with liquid nitrogen and were either immediately analyzed or stored at -80°C for up to a month.

Measurement of antioxidants

Glutathione and oxidized glutathione measurements were carried out according to

spectrophotometric procedure described by Knöerzer (130). Ascorbate was determined using HPLC according to Davey and associates (61) with modifications. Plant tissue was extracted in three volumes of 6% metaphosphoric acid containing 2mM EDTA and 1%PVPP. The extract was centrifuged for 12 minutes at 14,000 rpm at 5°C and supernatant was used for analysis. A Hitachi LaChrom Elite HPLC system (Hitachi High Technologies, USA) with an autosampler maintained at 6°C and a 120×5mm Synergi fusion column (Phenomenex, USA) packed with 4µm polar embedded C18 particles at 28°C were used. ASA was detected at 243nm using L-2420 UV-Vis Detector (Hitachi High Technologies, USA). The column was eluted with 1.0 $ml.min^{-1}$ flow of 5-30% acetonitrile in a mobile phase consisting of 400$µl.lit^{-1}$ orthophosphoric acid (pH 2.5). Analysis was completed in 12 minutes. Samples were then reduced by DTT (130) and analyzed by HPLC to determine total ascorbate (ASA+DHA). ASA concentration was deducted from total ascorbate to calculate the DHA concentration.

Enzyme activity measurement

Enzyme extraction and protein determination: Ground tissue was mixed with ten volumes of extraction buffer described by Knöerzer and associates (130). Addition of 1mM ASA to the extraction buffer was necessary to avoid inactivation of APX (11). The mixture was centrifuged at 14,000 rpm for 10 minutes at 5°C and the supernatant was used as the source of enzymes. Protein concentration was determined according to Bradford (40) with a standard curve prepared using bovines serum albumin.

Enzyme activity measurement: APX activity was assayed by decrease in absorbance at 290nm due to oxidation of ASA (130). MDAR activity was determined following the decrease in absorbance at 340 nm due to NADH oxidation as described by Hossain *et al.* (117). DHAR activity was determined following method described by Hossain

and Asada (116) by the decrease in DHA concentration at 265nm. GR activity was determined by increase in absorbance at 412nm according to Smith *et al*. (212). Pyrogallol-peroxidase (P-POX) activity was measured according to Knözer *et al*. (130) by increase at 430nm due to formation of purpurogallin from pyrogallol.

Gene expression

Total RNA was extracted using RNeasy plant mini kit (Qiagen, CA, USA) according to the manufacturer's instructions. DNase treatment was carried out using Turbo® DNase (Applied Biosystems, CA, USA) for 25 minutes at 37°C. One microgram of DNase-treated total RNA was used for cDNA synthesis using superscript™ III (Invitrogen, CA, USA) according to manufacturer's instruction. QRT-PCR analysis was performed with an iCycler iQ instrument using SYBR® green mastermix (Applied Biosystem, CA, USA) and gene-specific primers described in table 2.1. The PCR program consisted of an initial denaturation and Taq polymerase activation step of 5min at 95°C, followed by 40 cycles of 10s at 95°C and 1min at 60°C. The specificity of primers, lack of primer-dimer, and the absence of contaminating genomic DNA were verified, respectively, using amplicon dissociation curves, PCR in absence of cDNA, and by PCR analysis of RNA samples before reverse transcription. The amplification efficiency of primers, calculated using LinReg (11.0), was ≥ 80%, except for GR1, MDHAR2, DHAR1, cytosolic APX, Cytosolic CuZn-SOD (CuZnSODc), chloroplastid CuZn-SOD (CuZnSODp) with efficiencies ≥ %70. Expression levels were normalized using *actin* as reference gene and relative expressions of genes were calculated using the method of Ruijter and associates (178).

Statistical analysis

All experiments were designed as factorial 2×2 testing the effect of T22 or stress and

their interaction on growth and biochemical properties of seedlings. Analysis of variance (ANOVA) was performed using JMP 7 for Windows (SAS®) statistical package. Means were compared using a student's t-test.

Results

T22 treatment ameliorates the effects of paraquat and water deficit on seed germination and seedling growth

T22-treatment of seeds enhanced germination under water deficit or in presence of paraquat (Table 2.2 and Fig. 2.1*a*). In addition to its adverse effect on germination and seedling growth, paraquat induced change in pigmentation of tomato hypocotyls, which was not observed in T22-treated seedlings exposed to paraquat (Fig. 2.1*b*).

T22 treatment enhanced fresh and dry weight of tomato radicles and hypocotyls under water deficit compared to untreated seeds (Table 2.3). Chlorophyll content of hypocotyls as expressed by SPAD-units was enhanced by T22 treatment under both control conditions and water deficit (Table 2.3).

T22 treatment improves antioxidant capacity of tomato seedlings

Concentration and redox state of non-enzymatic antioxidants were analyzed in tomato radicles, the tissue where T22 colonizes (247), and hypocotyls 3 and 7 days after fifty percent of seeds germinated. T22 did not affect GSH concentration (P=0.8427), but the effect of T22×stress on GSH concentration was significant (P=0.0093). In other words, without water deficit, GSH concentration in hypocotyls of T22-treated seedlings was lower than control seedlings, but under water deficit GSH concentration of treated seedlings was higher than untreated seedlings (Fig. 2.2*a*). T22 treatment

Table 2. 1. Primers used for qRT-PCR analysis of genes encoding antioxidant enzymes in tomato

Gene code	Encoded enzyme	GI number	Primers	
			Forward	Reverse
GR1	Chloroplast glutathione reductase	167047069	ATAAAAATGCCGAGTTGCAG	GTTTACCATCCACATCCACTGT
GR2	Cytosolic glutathione reductase	220967701	AACTGGATGGCACCAAGAT	CTCTTCCAAGCTCAAGGCTT
DHAR1	Cytosolic dehydroascorbate reductase	66475035	TTCTGATGTCATTGTTGGGATTA	GCTCTTCGGAAATGAGACG
DHAR2	Chloproplast dehydroascorbate reductase	66475037	GAGGAGAAGTTCCCCAAACC	CGGAGTCTTTGCTTTTCAGG
MDHAR1	Chloroplast monodehydroascorbate reductase	110265125	GGAATCTTTGCCATTGGAG	GGAATCTTTGCCATTGGAG
APX2	Cytosolic ascorbate peroxidase 2	73761752	ACTATGCTAAGGCTCACTTGACA	CTAACGATATCCAACAATTCCAG
APX7	Stromal ascorbate peroxidase 7	74483952	CTTGCGAATTTTCATCGACA	GTGCCTCCTCGAGTGGTTAG
APX6	Thylakoid-bound ascorbate peroxidase 6	68300917	CGAAAGATGGACCAGGAAG	AAGAACAGCATCTGTAGGCAAA
CAT1	Catalase 1	170397	CTCAAACGCCTGTTATTTGTC	CGTGTCAGGGAACGACTTAG
CAT2	Catalase 2	5257184	AGACAAGAACGCTTTATTCGTC	GCCTAGATGCAAGCTTTTGA
FeSOD	Chloroplast Fe-superoxide dismutase	33439119	CTGGCTCTGCTACAATAACAGC	CCTGTGATACTTCCCCCAGT
Cu/Zn SOD 1	Cytosolic Cu/Zn-superoxide dismutase	170511	TGGTGATCTTGGTAACATCACA	TCCAATGATGGACTGTGGA
Cu/Zn SOD 2	Chloroplast Cu/Zn-superoxide dismutase	19192	AGGGTACAGTGGCTGTTGG	GTGAGTGGTCTTGCTCCGACT

significantly reduced GSSG concentration (*P*=0.010) and resulted in a higher GSH:GSSG ratio, whether seedlings were grown without or with stress.

Table 2. 2. Effects of water deficit, paraquat and T22 on germination of tomato

Stress	-T22	+T22	Statistical significance of effect [d]		
			T22	Stress	Interaction
-0.2 MPa[a]	75.1±4.3[c]	88.2±3.6	*	*	*
-0.4 MPa[b]	48.3±5.9	80.3+3.8	*	*	**
Paraquat (20μM)	72.3±4.4	81.9+5.7	n.s.	**	**

[a,b] Seedlings germinated in MS media containing 13.8 or 19.6% PEG 8000 respectively
[c]Germination percentage was measure 14 days after the start of imbibition
[d] n.s., *, ** respectively indicate non-significant effect or significant at α=0.05 or 0.01

Water deficit reduced GSH content of tomato hypocotyls (*P*=0.1271). Hypocotyls of untreated seedlings had significantly lower GSH than control, but GSH of treated seedling did not change significantly (Fig. 2.2*b*). GSH:GSSG ratio of untreated seedlings was slightly lower than control early after germination, but increased to control level a week later. T22 treated seedlings maintained GSH:GSSG levels higher than those of control seedlings throughout the experiment.

In radicles, T22 treatment did not change the GSH concentration (*P*=0.0891), however, it reduced GSSG (*P*=0.0011) and increased GSH:GSSG ratio compared to untreated seedlings (Fig. 2.2 *c*,*d*). Water deficit, increased concentration of GSH with or without T22 treatment (*P*=0.0352), but GSSG was only increased from that of control level in untreated seedlings. Therefore, GSH:GSSG ratio was enhanced in radicles of T22-treated seedlings under stress, but not in untreated seedlings.

Ascorbic acid is another important redox buffer and scavenger of ROS. T22 treatment, slightly, but not significantly, increased ASA concentration in tomato hypocotyls,

which together with significantly lower DHA concentration, resulted in higher ASA:DHA ratio compared to untreated seedlings (Fig. 2.3*a,b*). ASA concentration

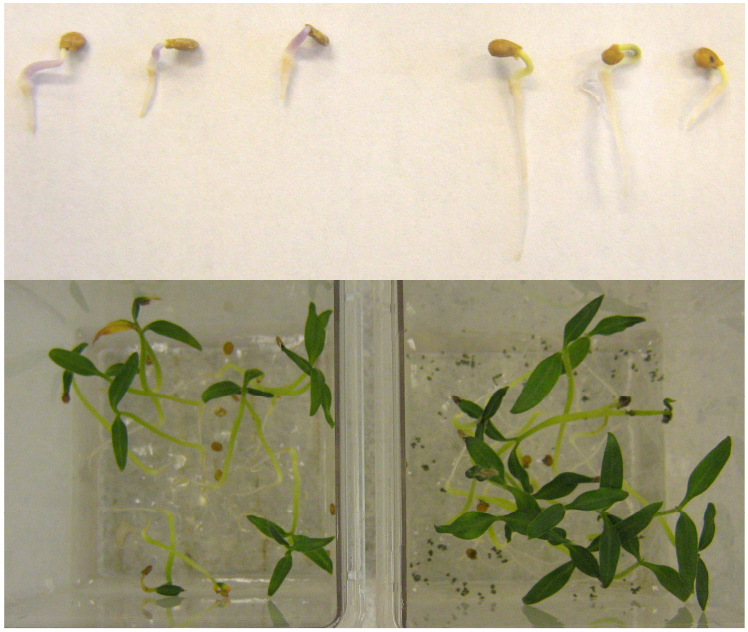

Figure 2. 1. Effect of T22-treatment and paraquat or water deficit on growth of tomato seedlings
Top left: 20µM Paraquat, top right: 20µM paraquat+T22
Bottom left: 13.8%PEG, bottom right T22+13.8%PEG

was reduced in response to water deficit, but DHA content was only increased in hypocotyls of untreated seedlings under stress, leading to lower ASA:DHA ratio in untreated seedlings, but not T22-treated seedlings (Fig. 2.3*a,b*). Time of sampling did not significantly affect ASA or DHA concentrations or the redox state of the molecule.

In radicles, T22 treatment slightly increased ASA concentration, but its main effect was not significant (Fig. 2.3*c*). The level of DHA was significantly smaller in radicles of treated plants and therefore, these radicles maintained higher ASA:DHA ratio (Fig 2.3*d*). Stress significantly changed the composition of ascorbate pool in roots with a significant reduction in ASA and increase in DHA resulting in lower ASA:DHA in

radicles from untreated seedlings, but not in radicles of treated seedlings (Fig. 2.3*c,d*).

Table 2. 3. Effect of *T. harzianum* and water deficit on biomass and chlorophyll content of tomato seedlings.

Hypocotyl (% of control)	-T22			+T22		Statistical significance of effect[f]		
	T22[e]	-0.2 MPa	-0.4 MPa	-0.2 MPa	-0.4 MPa	T22	Stress	Interaction
FW[ab]	-5.1	-17.2	-38.4	-3.1	-12.5	n.s.	*	**
DW[b]	-2.8	-22.9	-46.5	-5.4	-8.3	n.s	**	**
Chlorophyll content[c]	+3.9	-15.6	-21.5	-1.1	-5.0	*	*	*
Radicle (% of control)[d]								
FW	+3.1	-21.1	-45	-6.1	-10.0	n.s.	**	**
DW	-0.1	-29.7	-39	-9.3	-12.3	n.s.	**	**

[a] Values are expressed as percentage of those of untreated control seedlings, which were respectively 27mg, 2mg, and 39 SPAD units.
[b] Fresh weight (FW) and dry weight (DW) measured 14 days after the start of imbibition
c Chlorophyll content of fully developed hypocotyls measured 14 days after the start of imbibition
[d] Radicle dry weight is average of dry weight of all radicles pooled and dried together
[e] T22-treated seedlings germinated in MS media
[f] ns, *, ** respectively indicate non-significant effect or significant at α=0.05 or 0.01.

T22 treatment and water deficit affected the activity of Glutathione-Ascorbate cycle

The effect of water deficit, T22-treatment, and time of sampling on activity of Glutathione-Ascorbate cycle enzymes was analyzed. The activity of APX, was enhanced by both water deficit and T22-treatment. The greatest increase in the activity of the enzyme was observed in response to stress and T22 (Fig. 2.4). The activity of MDHAR, was only affected by water deficit and T22 treatment, with no major effect of time of sampling . The biggest increase in MDHAR activity was observed in T22-treated seedlings under stress. (Fig. 2.4). DHAR enzyme activity increased in response to T22, but not stress. Its activity reduced over time, but treated seedlings that were

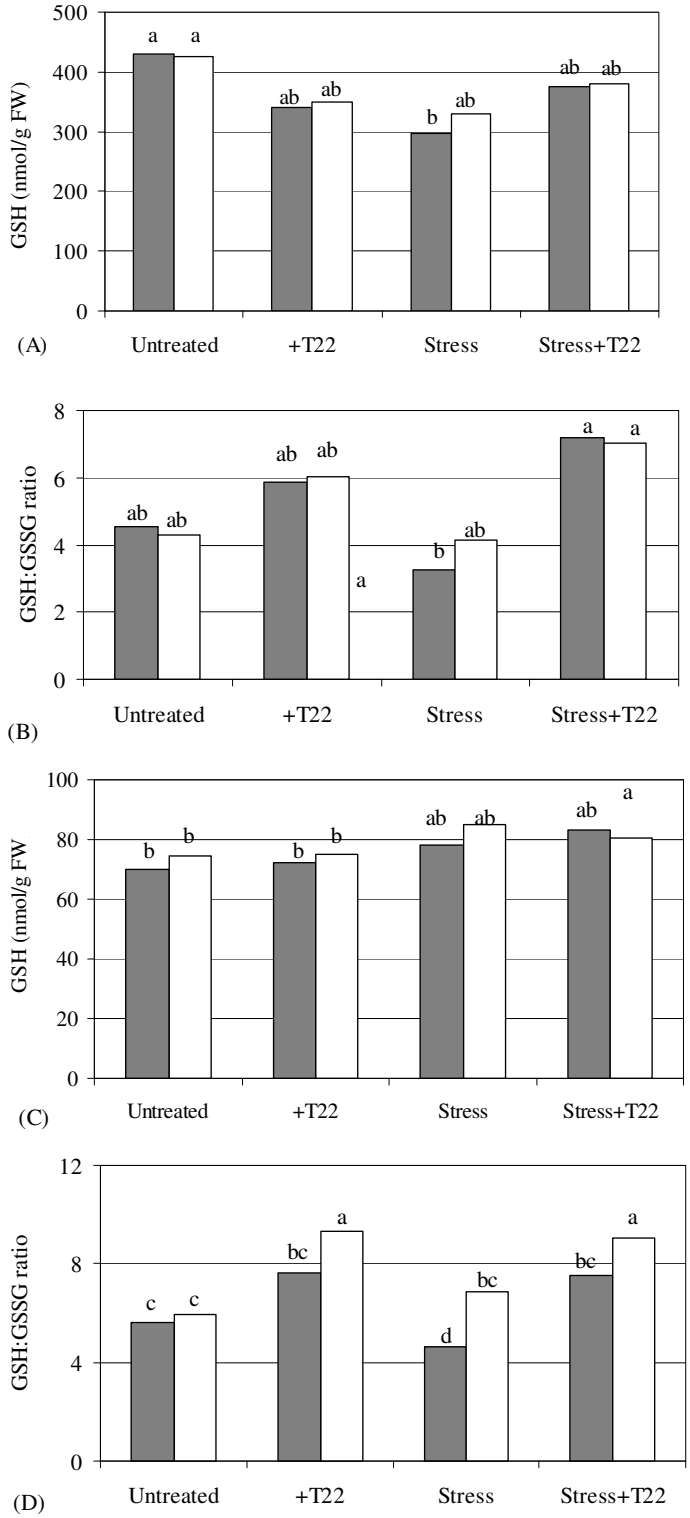

Figure 2. 2. (A) Concentration of reduced glutathione (GSH) and (B) reduced:oxidized glutathione (GSH:GSSG) in tomato hypocotyls and radicles (C-D) 3 or 7 days (gray or white bars) after fifty percent of seeds germinated.

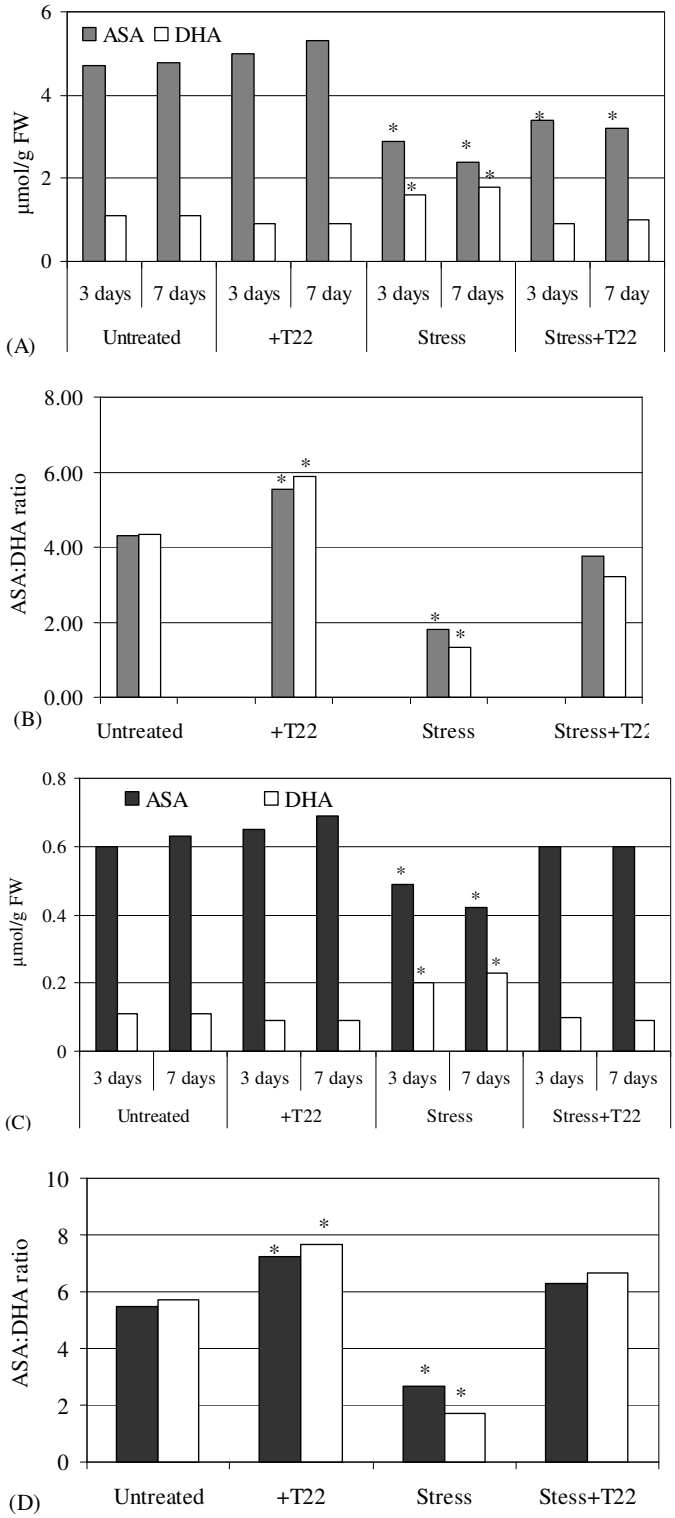

Figure 2. 3. Concentration of reduced (ASA) and oxidized ascorbate (DHA) in (A) hypocotyls and (C) radicles of tomato and ASA:DHA ratio in hypocotyls (B) and radicles (D) 3 (gray bars) and 10 (white bars) after 50% of seeds germinated

under stress had significantly higher DHAR activity than control (Fig. 2.4). The activity of GR, was enhanced by T22 treatment and water deficit (Fig. 2.4). The activity of GR continued to improve in T22-treated seedlings over time, but in untreated seedlings no furtherincrease in enzyme activity was observed (Fig. 2.4).

T22 increase pyrogallol-peroxidase (P-POX) activity (P=0.0006) of seedlings. Without water deficit activity of the enzyme was increased from 72.3±8.8 to 135.0±8.9 nmol.min^{-1}.mg^{-1} protein. In contrary, water deficit significantly reduced P-POX activity (P<0.0001). However, T22-tretaed seedlings maintained higher P-POX activity compared to control seedlings. The activity of P-POX continued to rise in T22-treated seedlings, but did not significantly change in untreated seedlings.

The relative expression of genes encoding glutathione-ascorbate cycle enzymes,

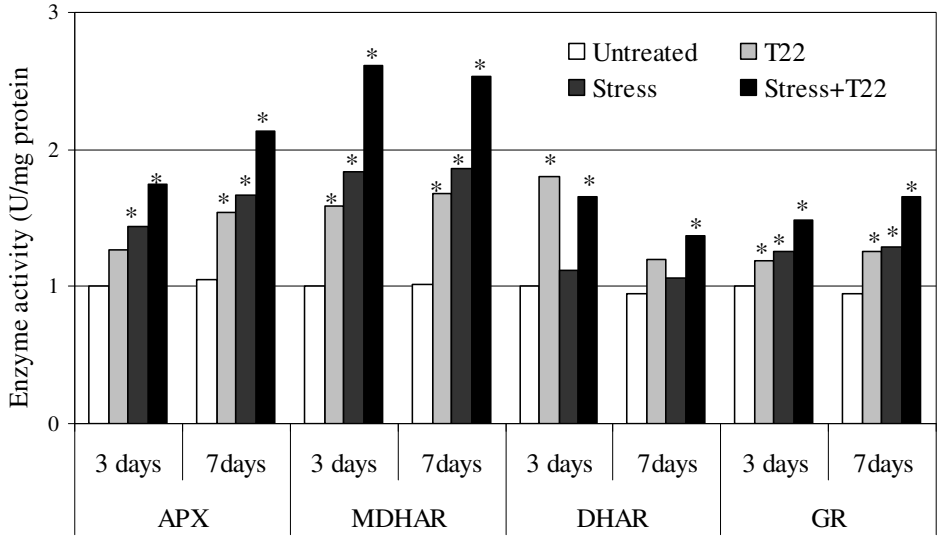

Figure 2. 4. Relative activity of ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase in tomato seedlings was affected by osmotic stress (addition of PEG to media), T22, and time of sampling.
Samples were taken 3 and 10 days after fifty percent of seedlings had emerged.
Enzyme activities were normalized to those of untreated seedlings 3 days after fifty percent of seeds had germinated, which respectively were 230.1, 204.3, 35.9, 29.6 nmol.min^{-1}.mg of protein for APX, MDHAR, DHAR, GR.

T22 affected expression of genes encoding antioxidant enzymes

catalases (CAT), and three superoxide dismutases (SOD) was determined in T22-treated seedlings three days after fifty percent of seeds germinated. T22-treatment enhanced the expression of genes encoding cytosolic APX (APXc), MDHAR1 and MDHAR2, both chloroplast and cytosolic DHAR (DHARp and DHARc) and both GRp and GRc. The relative expressions of thylakoid-bound or stromal APX (APXt and APXs) were not significantly affected by T22 treatment (Fig. 2.5*a*). The relative expression of chloroplast CuZn-SOD (CuZn-SODp) and Fe-SOD (Fe-SODp) significantly increased by T22, but not those of cytosolic CuZn SOD, CAT1 or CAT2 (Fig. 2.5*b*). The increase in relative expression of CuZn-SODp and GRp (6 times) followed by Fe-SODp> DHARc> APXc> MDHA2> MDHAR1> GRc> DHARp.

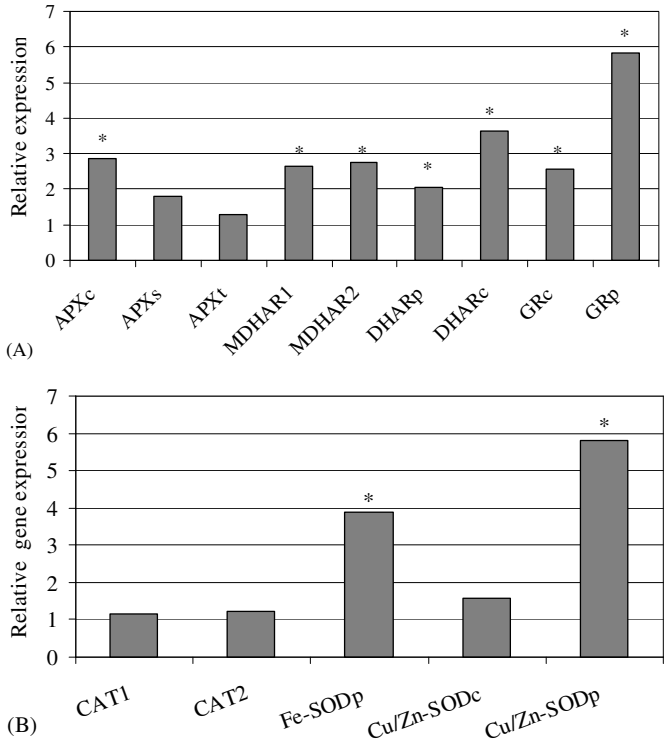

Figure 2. 5. Relative expression of genes encoding antioxidant enzymes in T22-treated seedlings.

Genes encoding isoforms of (A) cytosolic, thylakoid-bound, and stromal ascorbate peroxidase (APXc, APXt, APXs), Monodehydroascorbate peroxidase (MDHAP1,2), chloroplast and cytosolic dehydroascorbate reductase (DHARp, DHARc), and chloroplast and cytosolic glutathione reductase (GRp, GRc) and (B) catalases (CAT1, CAT2), chloroplast iron superoxide dismutase (FeSODp) and chloroplast and cytosolic copper/zing superoxide dismutase (Cu/Zn-SODp, Cu/Zn-SODc)

Discussion

Symbiotic relationships between *Trichoderma* spp. and wide range of host plants enhance growth and productivity under suboptimal conditions such as drought or water deficit (19, 98, 149, 202), salinity (251), oxidative damage (33), and unfavorable temperatures (33). In a recent report, we provided evidence that T22 enhances seed germination and seedling growth in a diverse set of abiotic and biotic stresses that otherwise negatively affected plants, suggesting involvement of a rather common and not stress specific defense mechanism. Therefore we focused on oxidative damage that could rise as a result of various abiotic stresses. Under water deficit, a negative correlation between T22 treatment and lipid peroxidation was observed (Mastouri, *et al.* unpublished), suggesting possible modification of antioxidant defense system. The current study clearly demonstrated that T22 treatment improved redox buffering capacity of seedlings by improving expression and activity of antioxidant enzymes.

T22 treatment modified dynamics of glutathione oxidation and reduction in hypocotyls and radicles of seedlings under stress. While untreated seedlings demonstrated a typical initial reaction to stress (217) with a reduction in GSH and GSH:GSSG ratio early after germination, followed by an acclimation phase characterized by increase in GSH:GSSG ratio (Fig 2.2*a,c*) no such reduction in

GSH:GSSG ratio of T22-treated seedlings was observed. It is possible that such a reduction in GSH:GSSG was delayed in T22-treated seedlings as at least in one study onset of drought response was delayed in *Trichoderma*-treated plants (19). Alternatively, such a change in the GSH:GSSG might not occur as T22-treatment induced higher antioxidant enzymes even in absence of stress (Fig. 2.3).

T22 treatment only slightly increased ASA concentration in tomato hypocotyls and radicles and no further increase in total ascorbate was observed under stress (Fig. 2.3), but the main difference between T22-treated and control seedlings was in the ASA:DHA ratio. While T22 increased the redox state of the ascorbate pool by maintaining most of the ascorbate in reduced form, even the moderate water deficit applied in this work resulted in significant oxidation of ascorbate pool. The reduction in ASA during stress, is due to its oxidation by APX to detoxify H_2O_2. In addition, the decline in the ASA may be also due to its role in regeneration of α-tocopherol or biosynthesis of zeaxanthin (211) both of which may increase in response to water deficit. Relatively higher activity of APX, MDHAR, DHAR, and GR in the T22-treated seedlings may elevate H_2O_2 scavenging and ASA regeneration capacity of seedlings leading to enhanced tolerance to abiotic stresses of diverse nature. In addition, the ability to maintain high ASA:DHA ratio and GSH:GSSG ratios, may maintain healthy plant growth as low ratio of reduced to oxidized form of both molecules diminish growth by blocking or delaying cell division and reducing protein synthesis (63). Consequent promotion in root growth may contribute to enhanced performance under water deficit as well (19, 98). Transgenic approaches to increase GSH content have shown that increase in total glutathione does not increase growth or tolerance to abiotic stresses and may even compromise growth due to oxidative damage (60, 246). In contrast, the importance of high activity of antioxidant enzymes

and maintaining high GSH:GSSH and ASA:DHA ratios for tolerance to water deficit or other abiotic stress has been confirmed using transgenic approaches (8, 9, 80, 150, 156) or through comparison of antioxidant enzyme activities of susceptible vs resistant plant varieties (142, 152, 153).

Osmotic adjustment is an alternative mechanism that may induce tolerance to abiotic stresses (211). It is an important mechanism of drought tolerance in plants associated with vesicular arbuscular mycorrhizae (VAM) that results in higher stomatal conductance (17). However, previous works with *Trichoderma* spp. (19) or other non-VAM endophytes (176) indicate no correlation between endophyte colonization, drought tolerance, and osmoticum concentration. In our works, we have not detected any effect of *Trichoderma* on stomatal conductivity (Mastouri, *et al.*, unpublished) that rules out the possibility of osmotic adjustment due to T22-treatment.

Relative expression of the several genes encoding antioxidant enzymes was increased in hypocotyls of tomato five days after the start of imbibition (Fig. 2.5), but only APXc gene was upregulated in radicles (data not shown) at this time. *Trichoderma* spp. colonize roots and remain limited to the cortex and outer layers of root epidermis (247), but the changes in the expression of antioxidant enzymes in hypocotyls (systemic tissue) were greater than radicles. *Trichoderma* induce systemic changes in gene expression through a complex signal transduction network (69, 70, 105, 202-204, 249) with MeJA playing the pivotal role as signaling molecule (204). Whether, this signaling network also stimulates the expression or activity of antioxidants is not known. However, it is known that MeJA induces expression of genes encoding antioxidant enzymes including GR (246). We found CGTCA-motif, a MeJA-responsive element (177), in promoter of majority of genes encoding antioxidant enzymes in *Arabidopsis thaliana* (data not shown), which suggests that MeJA may

play a signaling role in expression of genes encoding antioxidant enzymes as well. ROS and in particular H_2O_2 also stimulate expression of several antioxidant enzymes including GR, SOD, and GPX. We failed to detect any changes in the hydrogen peroxide in roots after treating them with conidial suspension of T22 (data not shown), but this could be because whole root was analyzed rather than the site of T22 penetration. However, at least two lines of evidence support that *Trichoderma* colonization may induce transient increase in ROS with signaling role. First, an increase in activity of POX and POX-mediated cross-linking of cell walls (247) has been observed in site of *Trichoderma* penetration in cucumber roots. Second, *Trichoderma*-cell free-culture filtrate is reported to induce a rapid increase in the ROS mediated fluorescence in soybean cell cultures (159). Such a signal may lead to activation of plant signaling cascade leading to upregulation of important families of defense related genes such as PR-proteins and antioxidant enzymes.

Evidences that were provided here support the hypothesis that *Trichoderma harzianum* strain T22, enhances the redox buffer capacity of plants, a trait that correlates well with tolerance to abiotic stresses. This phenomenon, if properly adapted, may enhance field performance of crop plants especially in marginal lands.

CHAPTER 3

Endophytic *Trichoderma* strains improve photosynthesis of tomato plants under water deficit and salinity

Abstract

Some fungi of *Trichoderma* genus improve tolerance of plants to abiotic stresses during germination or post-germination. We have recently shown that *T. harzianum* strain T22 improves antioxidant capacity of seedlings, especially under water deficit. T22 treatment upregulated expression of antioxidant enzymes, including two chloroplast superoxide dismutases and a chloroplast glutathione reductase (4-6 times higher expression in symbiotic plants as compared to non-symbiotic plants). The importance of these enzymes in water-water cycle and protection of chloroplasts from oxidative damage prompted us to examine the role of this symbiosis on protection of photosynthetic machinery during abiotic stresses. Five week old greenhouse grown tomato seedlings treated with *T. harzianum* strain T22, *T. virens* strain GV41 or untreated seeds, were subject to progressive water deficit or salinity. Water deficit was applied by withholding irrigation for 12 days and salinity by irrigating plants with 75mM NaCl solution for 7 days. Net photosynthesis rate (Pn) was measured only after salt treatment, but the chlorophyll fluorescence was measured as water deficit and salinity progressed. This data was automatically analyzed to determine F_v/F_m and PI_{ABS}, two commonly used parameters that correlate with efficiency of photosynthetic activity. Leaf area and shoot and root biomass (dry weight) were also measured. While salinity and water deficit reduced shoot biomass, the biomass of symbiotic plants was similar to control plants (unstressed plants). However, root biomass was only affected by stress. Pn reduced significantly after seven days of salt treatment, but plants treated with GV41 maintained higher rate of photosynthesis than both untreated and T22-

treated plants. Analysis of chlorophyll fluorescence dynamics (JIP test) showed that F_v/F_m was not sensitive enough to stress and was only reduced in untreated plants 12 days after withholding irrigation and 7 days after salt treatment. Gv41 and T22-treated plants maintained higher Fv/Fm values throughout both experiments. PI_{ABS} decreased as stress resulting from water deficit and salinity intensified, with bigger reductions in response to salinity. Symbiotic plants maintained higher PI_{ABS} under water deficit and salinity. GV41 was especially effective as PI_{ABS} of plants treated with this strain did not show any significant reduction in PI_{ABS} throughout both experiments. Analysis of component of PI_{ABS} showed additional differences in response of plants to the two strains of *Trichoderma*, which will be discussed in detail in this chapter.

Introduction

Some members of *Trichoderma* spp. have adapted symbiotic life style; they colonize roots of diverse group of monocot and eudicot hosts (34, 209, 247) and confer resistance to different biotic (103, 202) and abiotic stresses (19, 148, 149, 202, 251). These ubiquitous fungi are commercially available and their ability to enhance host tolerance to abiotic stresses may provide useful tools to improve agriculture especially in marginal areas. Therefore, understanding underlying mechanisms through which *Trichoderma* confers tolerance to abiotic stresses is a research priority.

Abiotic stresses increase production of reactive oxygen species (ROS) from the normal steady state (14, 151) and disrupt the balance between ROS production and scavenging by the antioxidant pathways (14, 77, 81, 150, 151, 161, 166, 211), causing an increase in the ROS level (14, 151) damage to photosynthetic machinery, inactivation of enzymes, peroxidation of lipids and damage to other macromolecules such as DNA (14, 151). Photosystem I and II (PSI and PS II) of chloroplasts of higher

plants are the major site of ROS production (14, 151). ROS are produced by direct transfer of excess energy to oxygen causing production of highly reactive singlet oxygen (PSII) (14, 157) or through transfer of electrons to O_2 in PSI resulting in formation of superoxide radicles (14). Abiotic stresses increases ROS production though both pathways by limiting photochemistry and disruption of electron transfer (14). Enzymatic and non-enzymatic antioxidants that maintain the balance between rate of production and detoxification of ROS in chloroplasts (14-16, 74) are the first line of defense against oxidative damage. We have recently reported that T22 treatment increases expression and activity of several antioxidant enzymes in tomato seedlings and improves redox state of non-enzymatic antioxidant molecules. Chloroplast isoforms of Cu/Zn-SOD and Fe-superoxide dismutase (Fe-SOD) and glutathione reductase (GR) were highly upregulated (4-6 times) in T22-treated seedlings in absence of stress. The role of these enzymes in detoxification of ROS in chloroplast, prompted us to ask whether this could enhance flow of electron in the photosystems and protect photosynthetic machinery under abiotic stresses. The main goal of this study was to determine whether the enhancement of antioxidant defense, was enough to protect photosynthetic machinery under water deficit and salinity, or whether other factors, such as modulation of stomatal aperture were involved.

Materials and methods

Plant and fungal material and seed treatment

Seeds of tomato (*Lycopersicum esculentum* L.) cultivar 'Jubilee' (Harris seeds, NY) were used. Fungal inoculants were prepared from *T. harzianum* strain T-22 (ATCC # 20847), hereafter T22, and *T. virens* strain GV41 (ATCC # 20906), hereafter GV41, coated onto cellulose and encapsulated with tapioca dextran (Crystal-Tex, National

Starch, NJ). To treat seeds a suspension of conidia in sterile type I water was prepared and seeds were treated with 20µl.g^{-1} of the conidial suspension to deposit 2×10^7 colony forming units per gram of seeds. Control seeds were treated with an equal amount of type I sterilized water. Seeds were planted directly and without air-drying in small containers. Water deficit experiment was carried out in fall 2009. Plants were watered three times a week with equal amount of water for four weeks. To impose water deficit, irrigation was stopped after four weeks for 12 days. Salinity experiment was carried out during spring 2010. Plants were irrigated with equal amount of water for each pot three times a week for three weeks and then seedlings were transplanted into bigger pots and were irrigated daily for one week. At the end of fourth week plants were irrigated daily with 500 ml of 75 mM NaCl solution for 7 days.

Photosynthetic measurements

Net photosynthesis (Pn) of salt stressed or unstressed plants was measured after 7 days of the start of salt treatment, using a PP Systems CIRAS-1 portable gas exchange system with a Parkinson broad leaf chamber (PP Systems, UK). Measurements were carried out in a growth chamber with 400µmol.m^{-2}.S^{-1} light at 25ºC on the youngest fully developed leaf of three plants from each treatment group in each replicate.

Leaf fluorescence measurement and JIP analysis

Fluorescence measurements were carried out between three to five hours after the start of photoperiod using a handy PEA fluorometer (Hansatech, USA). The instrument exposes dark adapted leaves to saturating levels of illumination (3000 µmol.m^{-2}.s^{-1}) that allows monitoring of the time dependent fluorescence induction kinetics known as Kautsky effect. Youngest fully developed leaf was dark adapted for 30 minutes before measurements and measurements were carried out within 12 days of withholding

irrigation or 7 days of salt treatment. After exposing leaves to saturating lights, the instrument calculates ground fluorescence (F_0) and measures maximal fluorescence (F_m), which are used to calculate variable fluorescence ($F_v=F_m-F_0$) and the ratio of variable to ground fluorescence (F_v/F_m). F_v/F_m, also known as potential quantum efficiency, is a commonly used indicator of potential efficiency of PSII and environmental stresses that affect the efficiency of PSII can significantly affect this indicator (32, 198). The performance index (PI_{ABS}) that is derived from the analysis of kinetics of rapid fluoresce (JIP test) is automatically calculated by the instrument. It is calculated as a function of the three main components that determine the potential photosynthetic activity by a PSII reaction center or (a) the density of reaction centers, (b) the quantum yield of the primary photochemistry, and (c) efficiency by which an electron moves from reduce quinone pool forward electron transfer as follows:

$$PI_{ABS} = \frac{RC}{ABS} \times \frac{\varphi_{P0}}{1-\varphi_{P0}} \times \frac{\psi_0}{1-\psi_0}$$

The handy PEA analyses the fluorescence curve automatically within two seconds and calculates PI_{ABS}. Detailed information about analysis of fast fluorescence curve and calculations made by the instrument are discussed by Strauss et al. (214).

PI_{CS}-performance index per cross section- which is another parameter that could be obtained from JIP test, was also calculated. To calculate this parameter, the density of reaction centers per cross section is used; in other words:

$$PI_{CS} = \frac{RC}{CS} \times \frac{\varphi_{P0}}{1-\varphi_{P0}} \times \frac{\psi_0}{1-\psi_0}$$

Biomass measurements

To estimate the effective canopy size, light interception and leaf area were measured.

Light interception was estimated by measuring the shaded area under plants when exposed to 60º light. Leaf area and light interception were highly correlated (R^2=0.985) and therefore, we will only report data from leaf area measurement throughout the text. Leaf area was determined using a li-3100C leaf area meter (Li-Cor, NE, US). Shoot (leaf and stem) fresh weights were measured and then roots and shoots were dried for 48 hours at 60ºC to determine root and shoot biomass.

Statistical analysis

The two experiments testing the response of plants to *Trichoderma* treatment and water deficit or salinity were conducted in fall 2009 and spring 2010. Each experiment was performed as a randomized block design with two blocks and three replicates in each block. Each replicate consisted of thirty plants, five plants per treatment. The two experiments were analyzed separately. Analysis of variance (ANOVA) was performed using JMP 7 for Windows (SAS®) statistical package. Means were compared using a student's t-test. Repeated measurements of chlorophyll fluorescence were subjected to multiple analyses of variance (MANOVA) and ANOVA.

Results

Photosynthesis and chlorophyll fluorescence measurements

Seven days after irrigating plants with 75 mM NaCl, net photosynthesis (Pn) decreased significantly. However, fungal inoculation enhanced photosynthesis (Table 3.1). Leaf conductance, transpiration and internal/external CO_2 of salt-stressed plants were significantly lower compared to unstressed plants, however, effect of fungal inoculation or stress × fungal inoculation were not significant (Table 3.1).

Leaf fluorescence measurement and JIP analysis

The main effect of stress on F_v/F_m was not significant, indicating that this value is not sensitive to either stresses. Although symptoms of stress, wilting and necrotic spots started to appear within 3 days of salt treatment and 5 days of withholding irrigation, F_v/F_m declined only 12 days after withholding irrigation and 7 days after salt treatment (Table 3.2 *a,b*). Fungal inoculation, in contrast, significantly improved F_v/F_m, with GV41-treated plants showing significantly higher F_v/F_m.

PI_{ABS} decreased as water deficit and salinity stresses intensified (Table 3.3 *a,b*). However, fungal inoculation improved the PI_{ABS} under salinity or water deficit. There was a significant difference between plants treated with two strains of *Trichoderma* in terms of the response of their PI_{ABS} to stress; GV41-treated plants maintained significantly higher PI_{ABS} than T22-treated plants (Table 3.3 *a,b*) in both experiments.

Table 3. 1. Photosynthesis and other related physiological variables of symbiotic or non-symbiotic plants in response to salinity

Treatment	Pn[c]	Percentage of control	E (mmol. $m^{-2}.S^{-1}$)[d]	g (mmol. $m^{-2}.S^{-1}$)[e]	C_i/C_a ($ppm.ppm^{-1}$)[f]
Control [a]	6.42±0.31[g]	-	2.85±0.22	137±22.6	0.67±0.02
GV41[a]	6.78±0.23	8.7	2.81±0.19	151±19.2	0.69±0.01
T22[a]	7.28±0.36	13.4	2.93±0.11	142±20.5	0.70±0.03
Stress[b]	4.06±0.18*	-36.8	1.77±0.15*	70±23.5*	0.56±0.02
Stress+GV41	4.84±0.27*	-24.6	1.49±0.13*	66±16.8*	0.59±0.02
Stress+T22	4.97±0.23*	-22.6	1.59±0.13*	68±21.3*	0.58±0.01
Statistical significance (*P-value*)					
Stress	<0.0001		<0.0001	<0.0001	<0.0001
Inoculation	0.0428		0.4561	0.5922	0.1707
Stress ×Inoculation	0.7163		0.3317	0.5210	0.6998

[a] Untreated, treated with *T. virens* strain GV41 or *T. harzianum* strain T22
[b] Salt stress applied by irrigating five week-old plants with 75mM NaCl for one week
[c] Net photosynthesis or photosynthesis per unit leaf area per second ($\mu mol.m^{-2}.s^{-1}$)
[d] Transpiration rate
[e] Leaf conductivity
[f] Internal CO_2/atmospheric CO_2 concentration
[g] Each number is average of eighteen measurements± standard error of mean

Component of PI_{ABS} were also analyzed. The ratio of chlorophylls in the reaction centers to the chlorophylls in the antenna (RC/ABS) increased in response to stress ($P \leq 0.0001$ in both experiments). The effect of stress× inoculation was significant ($P=0.0155$ in water deficit experiment and $P=0.0349$ in salinity experiment) and the biggest increase was observed in the GV41-treated plants (Fig. 3.1 *a,b*). The second component related to the maximum yield of primary photochemistry ($\varphi_{P0}/1-\varphi_{P0}$), did not change in response to stress, but symbiotic plants, especially GV41-treated plants had higher $\varphi_{P0}/1-\varphi_{P0}$ than untreated plants (Fig. 3.1 *a,b*).

Table 3. 2. Potential quantum efficiency (F_v/F_m) of symbiotic or non-symbiotic plants as affected by (a) water deficit or (b) salinity

(a)	Days after withholding irrigation				
Treatment	0	2	4	7	12
Control [a]	0.7798[c]	0.7756	0.7705	0.7697	0.7723
GV41[a]	0.7993	0.7899	0.7915	0.7957	0.7889
T22[a]	0.7879	0.7879	0.7803	0.7877	0.7839
Water Deficit (WD)[b]	-	0.7641	0.7638	0.7751	0.7512*
WD+GV41	-	0.7748	0.7958	0.7957	0.7960
WD+T22	-	0.7790	0.7943	0.7756	0.7818
Statistical significance (P-value)					
Stress	0.6137				
Inoculation	0.0205				
Stress× Inoculation	0.0893				

(b)	Days after start of salt treatment			
Treatment	0	2	4	7
Control	0.7853	0.7756	0.7705	0.7871
GV41	0.7838	0.7949	0.7915	0.7938
T22	0.7998	0.7907	0.7895	0.7863
Salinity [d]	-	0.7692	0.7689	0.7602*
Salinity+GV41	-	0.7859	0.7892	0.7901
Salinity+T22	-	0.7856	0.7803	0.7889
Statistical significance (P-value)				
Stress	0.3325			
Inoculation	0.0375			
Stress× inoculation	0.1453			

[a] Untreated, treated with *T. virens* strain GV41 or *T. harzianum* strain T22
[b] Water deficit applied by withholding irrigation for 12 days
[c] Each value is average of thirty measurements
[d] Salinity was applied by irrigating plants with 75 mM NaCl
* Averages are statistically different form those of untreated control plants

The other component of PI_{ABS} that relates to the efficiency of electron transfer ($\phi_{P0}/1-\phi_{P0}$) decreased in response to stress, but symbiotic plants, especially GV41-treated plants maintained higher $\phi_{P0}/1-\phi_{P0}$ than untreated plants under both salinity and water deficit (Fig. 3.1 *a,b*). Concentration of reaction centers per cross section (RC/CS) was only affected by the stress × inoculation effect. Under both salinity and water deficit, GV41-treated plants had significantly higher RC/CS, followed by T22-treated plants (Fig. 3.1 *a,b*).

Table 3. 3. Performance index per absorbance (PI_{ABS}) of symbiotic or non-symbiotic tomato plants as affected by (a) water deficit or (b) salinity

(a)	Days after irrigation				
Treatment	0	2	4	7	12
Control [a]	1.30[c]	1.42	1.09	1.29	1.08
GV41 [a]	1.35	1.32	1.45	1.34	1.52
T22 [a]	1.60	1.26	1.36	1.47	1.28
Water Deficit (WD)[b]	-	0.78*	0.80*	1.02	0.76*
WD+GV41	-	1.35	1.36	1.21	1.15
WD+T22	-	1.00	1.50	0.99	0.82*
Statistical significance (P-value)					
Stress	0.0007				
Inoculants	0.0425				
Stress× inoculant	0.0364				

(b)	Days after irrigation			
Treatment	0	2	4	7
Control	1.52	1.28	1.13	1.21
GV41	1.56	1.39	1.29	1.24
T22	1.49	1.01	0.99*	0.86*
Salinity[d]	-	0.61*	0.72*	0.87*
Salinity+GV41	-	1.35	1.39	1.07
Salinity+T22	-	1.32	1.29	1.06
Statistical significance (P-value)				
Stress	0.0021			
Inoculants	0.0425			
Stress× Inoculant	0.0512			

[a] Untreated, treated with *T. virens* strain GV41 or *T. harzianum* strain T22
[b] Water deficit applied by withholding irrigation for 12 days
[c] Each value is average of thirty measurements
[d] Salinity was applied by irrigating plants with 75 mM NaCl
* Averages are statistically different form those of untreated control plants

Biomass accumulation

Leaf area was measured in the salinity experiment. Leaf area was reduced by stress (P<0.0001), but the salinity × seed inoculation effect was significant. GV41-treated plants had significantly higher leaf area than T22-treated plants and control when treated with salt water.

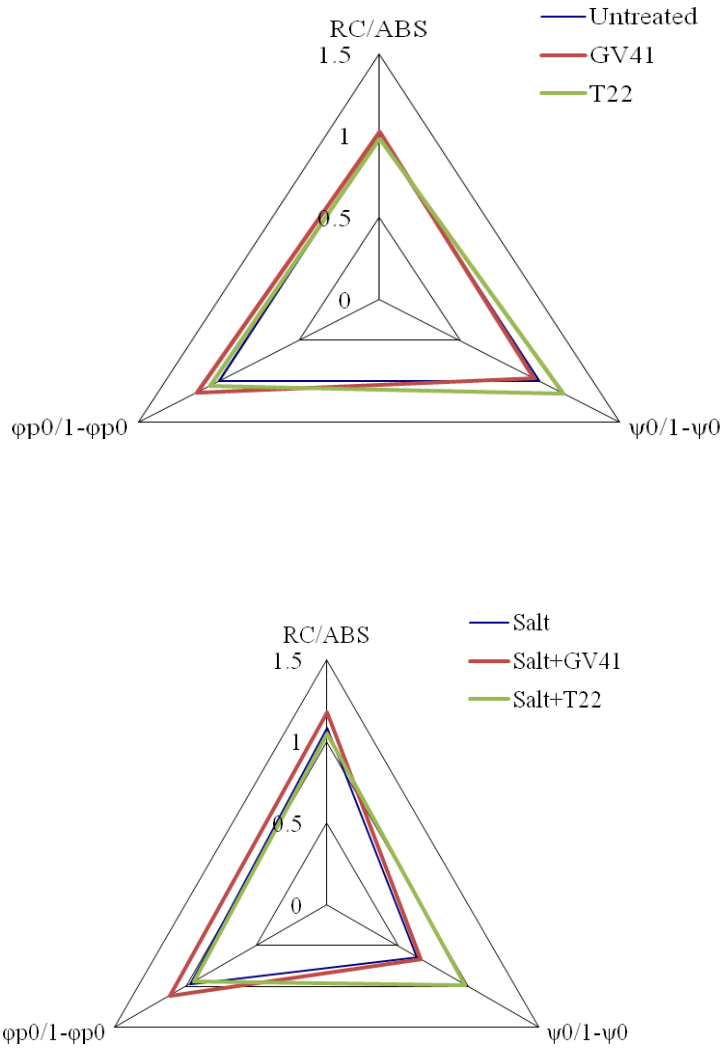

Figure 3. 1. Radar plots depicting changes in the components of PI_{ABS} affected by inoculation with (a) *Trichoderma* strains and (b) salinity stress.

Salinity and osmotic stress significantly reduced biomass (DW) of shoots and roots (Table 3.4). Seed inoculation with symbionts positively affected shoot biomass in both experiments, but there was no effect of this treatment on root biomass (Table 3.4). Shoot: root ratio was not affected by salinity, water deficit or fungal inoculation.

Discussion

Symbiotic fungi *Trichoderma harzianum* strain T22 and *T. virens* strain GV41 increased biomass of tomato plants under salinity and water deficit (Table 3.4 *a,b*). These stresses can limit the rate of photosynthesis by stomatal closure (58), non-stomatal biochemical impairment of carbon assimilation (47, 58, 165, 219) and biochemical damage to photosynthetic machinery (65). This research was an attempt to determine whether symbiosis with *Trichoderma* spp. can increase rate of photosynthesis under abiotic stresses and whether this is due to better protection of photosynthetic machinery or other factors.

Results presented here demonstrated that symbiotic plants maintained higher Pn under salinity, which could not be explained by differences in leaf conductivity (g) (Table 3.1) as it is suggested for vesicular arbiscular mycorrhizae (VAM)- mediated drought tolerance (17, 216). However higher Pn of symbiotic plants under stress was parallel to higher PI_{ABS} (Table 3.3), suggesting that the higher Pn of symbiotic plants was related to higher efficiency of PSII during stress (253). We found a close correlation between Pn and PI_{ABS} which indicates that this parameter can be reliably used to monitor the performance of plants under salinity and drought conditions. Previous works on a variety of photosynthesizing organisms also has indicated the sensitivity of PI_{ABS} as indicator of abiotic stresses (134, 164, 227).

In contrary, F_v/F_m ratio, a measures the potential quantum efficiency of PSII (32), did

Table 3. 4. Growth of symbiotic or non-symbiotic tomato seedlings as affected by (a) water deficit or (b) salinity

Treatment	Leaf area (cm^2)	Total shoot DW[c] (g)	Root DW (g)	Shoot:Root (g:g)
Control[a]	-	5.2±0.21[d]	1.01±0.07	5.1±0.5
GV41	-	5.7±0.28	0.97±0.09	6.0±0.4
T22[a]	-	5.6±0.35	1.10±0.08	5.2±0.6
Water deficit (WD) [b]	-	4.0±0.21*	0.83±0.09	4.8±0.3
WD+GV41	-	5.2±0.29	0.91±0.10	5.7±0.4
WD+T22	-	4.9±0.28	0.89±0.09	5.5±0.4
Statistical significance (*P-value*)				
Stress		<0.0001	0.0219	0.5982
Inoculation		0.0301	0.4388	0.6024
Stress × Inoculation		0.6549	0.1229	0.3723

(b)

Treatment	Leaf area (cm^2)	Total shoot DW (g)	Root DW (g)	Shoot:Root (g:g)
Control	1467.5±44.2	7.1±0.32	1.48±0.09	5.0±0.4
GV41	1332.4±47.1*	7.4±0.34	1.29±0.07	5.7±0.3
T22	1299.5±51.0*	8.1±0.38*	1.63±0.12	5.0±0.2
Salt stress[e]	906.8±47.1*	5.5±0.33*	1.04±0.11	5.3±0.2
Salt+GV41	978.9±40.4*	6.1±0.32	1.26±0.10	4.8±0.3
Salt+T22	948.4±42.1*	6.4±0.30	1.03±0.09*	6.2±0.4
Statistical significance (*P-value*)				
Stress	<0.0001	<0.0001	0.0168	0.7187
Inoculation	0.0611	0.0395	0.8986	0.9901
Stress × Inoculation	0.0030	0.9225	0.2333	0.2591

[a] Untreated, treated with *T. virens* strain GV41 or *T. harzianum* strain T22
[b] Water deficit applied by withholding irrigation for 12 days
[c] Dry Weight
[d] Each number is average of eighteen measurements± standard error of mean
[e] Salinity was applied by irrigating plants with 75 mM NaCl

not significantly change as water deficit and salt stresses intensified and only slight reduction in F_v/F_m values were observed after 12 days of withholding irrigation or 7 days of salt treatment in untreated plants. This is consistent with previous reports that mild water deficit or salinity do not affect the efficiency of the primary photochemical events of PSII or the associated fluorescence induction parameters such as F_v/F_m (26, 227, 243). During water deficit or salinity, water-water cycle (15, 16, 74) in PSI maintains a constant sink for the electrons and prevent inactivation of PSII, thereby

buffers changes in F_v/F_m. Sever stress and excess light, on the other hand, can significantly reduce F_v/F_m (157, 198, 243). For instance, when Arabidopsis plants adapted to 70μmol.m^{-2}.s^{-1} were exposed to high light (≥400μmol.m^{-2}.s^{-1}) the F_v/F_m was reduced to 0.45 (149, 202). However, even under those circumstances T22-treated plants maintained relatively high F_v/F_m value of 0.75. Our recent observation that T22 increases the expression of enzymatic antioxidants may help to explain how symbiotic plants maintained higher F_v/F_m values under stress. Similarly, several other reports have also correlated the enhancement in antioxidant capacity of symbiotic plants to their better performance under abiotic stresses (27, 89, 176, 237).

Symbiotic *Trichoderma* spp. often improve root growth, root length density (RLD) or lateral root formation (19, 33, 35, 36, 57, 109). This could significantly improve uptake of water and nutrients (98) and improve tolerance to water deficit and salinity. However, in this work symbiosis was not associated with an increase in root biomass (Table 3.4) or root area (data not shown). This could be due to the limitation imposed by limited pot size that restricted root growth. Nevertheless, this situation provided us with an opportunity to investigate the role of symbionts on abiotic stress tolerance, separate from modified root growth and allowed us to conclude that tolerance to abiotic stresses could occur independent of root promotion. However, this data does not undermine the importance of symbiotic-related root promotion in field conditions, where access to diminishing water resources can maintain growth.

This work was an attempt to investigate the effects of symbiosis between two strains of *Trichoderma* spp. on photosynthesis under salinity and water deficit. These two sources of stress were chosen due to the ever increasing pressure of salinity and water deficit on crop production worldwide. Results described here demonstrated that the symbionts of this genus show substantial promise to improve crop production when

water scarcity or soil and irrigation water salinity limit crop production. We recognize that more field trials are required to evaluate the capacities of these fungi. The complication in the field may arise due to constant and substantial temperature fluctuation, which can significantly affect plant-water relations and inactivate PSII (30, 172) or other sources of stress including biotic stresses. However, the biocontrol abilities of these fungi (103) and their role in enhanced plant nutritional status (10, 250) together with enhanced tolerance to abiotic stresses, may extend the duration of photosynthesis which has shown tight connection to productivity (173).

CHAPTER 4

Induced systemic resistance and plant responses to fungal biocontrol agents

Abstract

Biocontrol fungi (BCF) are agents that control plant diseases. These include the well-known *Trichoderma* spp. and the recently described *Sebacinales* spp. They have the ability to control numerous foliar, root, and fruit pathogens and even invertebrates such as nematodes. However, this is only a subset of their abilities. We now know that they also have the ability to ameliorate a wide range of abiotic stresses, and some of them can also alleviate physiological stresses such as seed aging. They can also enhance nutrient uptake in plants and can substantially increase nitrogen use efficiency in crops. These abilities may be more important to agriculture than disease control. Some strains also have abilities to improve photosynthetic efficiency and probably respiratory activities of plants. All of these capabilities are a consequence of their abilities to reprogram plant gene expression, probably through activation of a limited number of general plant pathways.

Introduction

Biocontrol fungi (BCF) are beneficial organisms that reduce the negative effects of plant pathogens and promote positive responses in the plant. Recent data indicates that their abilities to control plant diseases are only a subset of their capabilities. They do control diseases and in addition have other benefits, including amelioration of intrinsic physiological stresses in seeds and alleviation of abiotic stresses. They can also improve photosynthetic efficiency, especially in plants subjected to various stresses. Finally, several fungi also increase nitrogen use efficiency in plants. As a

consequence, plants treated with beneficial fungi may be larger and healthier and have greater yields than plants without them. Mechanisms by which these changes occur are becoming known.

Most of the early work on biocontrol of plant diseases by *Trichoderma* spp. revolved around the direct ability of these fungi to interact with soil pathogens. The specific mechanisms described were mycoparasitism, production of antibiotics, and competition for nutrients in the rhizosphere (50, 103, 110, 232). During the process of mycoparasitism, the fungi first locates target hyphae by probing with constitutively produced cell wall degrading enzymes (CWDEs) coupled with very sensitive detection of cell wall fragments released from target fungi (103, 208, 233, 252). Expression of fungitoxic CWDEs is induced, and these diffuse toward the target fungi and attack even before physical contact (42, 235, 252). This detection stimulates increased and directional growth toward the target fungus (42, 235, 252). Once the fungi come into contact, *Trichoderma* spp. attach and may coil around and form appresoria on the surface of the host (124). Enzymes and antibiotic substances are produced that kill and/or degrade the target hyphae and permit penetration of the *Trichoderma* strains. Both the enzymes and the antibiotics are strongly antifungal and are synergistic in their action (51, 118, 143, 189).

However, more recent findings indicate that a primary method of pathogen control occurs through the ability of the fungi to reprogram plant gene expression. As a consequence, induced systemic resistance (ISR) occurs. Genetic reprogramming also induces mechanisms in the plant that alleviate physiological and abiotic stresses and to improve plant nitrogen use efficiency (NUE). Significant progress in understanding how BCF interact directly with the plants has been achieved. This review describes

new knowledge regarding BCF's abilities and the molecular mechanisms for induction of plant responses.

Formation of the interaction and local plant responses

Many *Trichoderma* strains colonize plant roots of dicots and monocots (95). During this process *Trichoderma* hyphae coil around the roots, form appresoria-like structures, and finally penetrate the root cortex (247). *Trichoderma* grows intercellularly in the root epidermis and cortex and induces the surrounding plant cells to deposit cell wall material and produce phenolic compounds. This plant reaction limits the *Trichoderma* growth inside the root (247). *Piriformospora indica*, the model system for *Sebacinales* fungi, has root colonization characteristics different from that of *Trichoderma* spp. These axenically culturable mycorhiza-like fungi (230) colonize the root elongation zone mainly intercellularly. However, the root differentiation zone is heavily infested by inter- and intracellular hyphae, and the majority of the hyphae are present in dead rhizodermal and cortical cells, which become completely filled with chlamydospores (66). *P. indica* seems to induce cell death by interfering with the host cell death machinery (66) and not by releasing cytotoxic molecules (187). Nevertheless, this does not provoke root-tissue necrotization and does not resemble pathogen-derived programmed cell death (66, 187, 188, 193, 237). Unlike *Trichoderma*, growth of *P. indica* within the root cortex does not induce visible cell wall reinforcement (188, 213, 238). *P. indica* may even induce production of gibberellins as part of modulation of plant defenses in the roots (188). Other mechanisms are employed by the plant to restrict the growth of this fungus within roots, and recent studies implicate a β-glucosidase, PYK10, and perhaps germines in this process (188, 198), as well as salicylic acid (213). When inside plant roots, fungi have access to plant nutrients, which allow them to proliferate. Moreover, they

significantly enhance root growth in many cases (71, 92, 98, 109, 167, 224), thus providing more niches for growth of the fungi. The plants benefit from this relationship through increased root and shoot growth, increased macro- and micronutrient uptake, and protection from diseases (84, 93, 103, 193, 197, 206, 247, 250). This interaction of BCF with the plant results in reprogramming plant transcriptome and proteome (6, 146, 188, 191, 200, 201, 238). Hence, this interaction is mutually beneficial. Given that *Trichoderma* spp. and other fungi are also capable of living freely in soil, they should be considered as opportunistic plant symbionts.

Fungal compounds involved in induction of plant responses

Studies revealed many classes of compounds that are released by *Trichoderma* spp. into the zone of interaction and induce resistance in plants. The first class is proteins with enzymatic or other activity. Fungal proteins such as xylanase, cellulase, and swollenin are secreted by *Trichoderma* species (12, 82, 144, 147) but seem to induce only localized plant reactions and necrosis (25, 41, 147). *Trichoderma* endochitinase can also enhance defense, probably through induction of plant defense–related proteins (110, 143). Other proteins and peptides that are active in inducing terpenoid phytoalexins biosynthesis and peroxidase activity in cotton, e.g., the small protein, SM1, which has hydrophobin-like properties, were found to be produced by strains of *T. virens* (68, 70, 90). Another hydrophobin-like protein produced by T22 that induces both enhanced root development and disease resistance was identified (179). Another group of proteins that induce defense mechanisms in plants are the products of avirulence-like (Avr) genes (244, 245). These are not only produced by a variety of fungal and bacterial plant pathogens but also by BCF. They usually function as race- or pathovar-specific elicitors of hypersensitive and other defense-related responses in plant species that contain the corresponding resistance (R) gene. At least some of these

fungal elicitors of plant defense response could be identified by plants as microbe-associated molecular patterns (MAMPs). This recognition plays a key role in innate immunity (29). A different group of metabolites that induce plant defense mechanisms against pathogens are peptaibols. Peptaibols are a class of linear short-chain length (≤20 residues) peptides of fungal origin produced by the nonribosomal peptide synthase. The biological role of peptaibols has been demonstrated in few systems, and antimicrobial activity was reported (55, 56, 189, 215, 241). However, a growing number of reports indicate that peptaibols can elicit plant defense responses (48, 73, 236).

Another class of elicitors of plant defense includes oligosaccharides and low-molecularweight compounds. These are released from fungal or plant cell walls by the activity of *Trichoderma* enzymes (103, 244, 245). Other small secondary metabolites produced by different *Trichoderma* strains were also isolated and shown to induce expression of pathogenesis related (PR) proteins when applied to plants as well as reduce disease symptoms systemically (232). Less-characterized metabolites produced by other BCF induce resistance, induce lignifications at the site of pathogen infection, and elicit generation of reactive oxygen species (ROS) (67). Plant responses were also recorded for a cell wall extract from *P. indica*. However, these extracts promote growth but not defense responses (225). It appears that modulation of Ca^{2+} signal perception as well as H^{+}signaling are an early step of plant cells response to the interaction with BCF metabolites (76, 159, 225).

Increased disease resistance

Although mycoparasitism was considered to be highly important in many systems, antibiosis was the accepted mechanism for others such as the biocontrol of

Rhizocontia solani by *T. virens* (118). However, a series of mutations in *T. virens* resulting in deficiency of mycoparasitic ability and/or inability to produce antibiotics had no effect on the biological activity of these strains (121). Instead, there was a very strong correlation between the abilities of these strains to induce terpenoid phytoalexins defense compounds in cotton seedlings and control of *R. solani.* Another classical biocontrol using *Trichoderma* spp. has been the control of seed rotting *Pythium* spp. by *T. harzianum* strain T22 (106, 123), in which mycoparasitism was considered the primary mechanism. However, it was recently demonstrated that control of *P. ultimum* on *Arabidopsis* seedlings by T22 required the *NPR1* gene, which is a key gene involved in disease resistance (F. Mastouri & G.E. Harman, unpublished data). These examples clearly demonstrate the importance of induction of plant responses by BCF. In other plant-pathogen systems, the effect clearly is via plant systemic response because BCF and pathogen are spatially separated (in dicotyledonous and monocotyledonous plants). Thus, any effect must be via systemic resistance. A range of pathogens were found to be controlled from fungi to oomycetes to bacteria and even one virus (3, 31, 62, 103, 109, 132, 141, 193, 213, 237, 239, 249). Systemic changes are frequently associated with enhanced levels of PR proteins and/or with accumulation of phytoalexins type compounds (3, 68, 109, 137, 188, 239, 248, 249). *Trichoderma* spp. are not the only well-documented fungi that induce systemic resistance. *P. indica* has very similar capabilities (213, 239). Essentially, the data regarding induced resistance have dealt with disease control, but there is a good prospect that these systems may also increase resistance to, or enhance predation of, insect pests, especially because the ethylene/ jasmonate pathway is involved in plant resistance to insects (190, 222). Similar pathways, such as the jasmonate/ethylene pathway of induced resistance, are induced by insect herbivory, so if this effect was enhanced by the presence of *Trichoderma*, then greater insect control would probably

result. In addition, as described above, *Trichoderma* spp. have abilities to limit nematode damage (95, 195, 196). However, *Sebacinales* may increase growth performance at the expense of herbivore resistance (28).

Plant signaling pathways induced by BCF leading to disease resistance

Contact with pathogenic and nonpathogenic microorganisms triggers a wide range of defense mechanisms in plants. Two main mechanisms are recognized: systemic acquired resistance (SAR) and induced systemic resistance (ISR). SAR is usually triggered by local infection, provides long-term systemic resistance to subsequent pathogen attack, is correlated with the activation of PR genes, and requires the involvement of the signal molecule salicylic acid (SA) (73). ISR is known to result from colonization of roots by certain nonpathogenic rhizosphere bacteria (229). ISR is not SA-dependent, but rather requires components of the jasmonic acid (JA) signaling pathway followed by the ethylene signaling pathway.

The molecular mechanisms activated by *T. asperellum* in cucumber have been particularly well studied. Colonization of *T. asperellum* on roots induces resistance to *Pseudomonas syringae* pv. *lachrymans* (*Psl*) on foliage. During the process of the *Trichoderma* interaction with the plant, SA content did not differ from that of control plants, even though *Psl* infection did increase salicylate concentrations . However, the biocontrol activity of the organism was strongly reduced by diethyldithiocarbamic acid (DIECA), an inhibitor of JA production, or silver-thiosulfate (STS), an inhibitor of ethylene activity. These data strongly suggested that both JA and ethylene are required for the biocontrol activity of the fungi. Neither treatment affected colonization of roots by the fungus. However, ethylene content did not differ between control and *Trichoderma* inoculated plants, suggesting that although ethylene

signaling is required, total ethylene levels did not change (204). This is similar to the finding that JA and ethylene are involved in ISR induced by rhizobacteria (168).

Further evidence for the involvement of JA and ethylene in transducing the signals from *Trichoderma*-inoculated roots to the leaves comes from gene expression studies. In roots, real-time reverse transcription polymerase chain reaction (RT-PCR) indicated that *Lox1*, which encodes a lipoxygenase involved in jasmonate synthesis and controls a feed-forward loop in jasmonate synthesis, was upregulated by inoculation with *T. asperellum. Lox1* is induced in the roots as early as 1 h post *Trichoderma* inoculation. A second peak was observed 24 h post-inoculation, possibly resulting from the initiation of the octadecanoic pathway and the synthesis of JA. Another gene found to be upregulated by *Trichoderma* inoculation is *Pal1*, which encodes for phenylalanine ammonia-lyase (PAL) (201, 204). Activity of PAL was also shown to increase in sunflower by *Trichoderma* inoculation (137). *Pal1* is considered to be activated by JA/ethylene signaling during plant defense response. It catalyzes the first step of phenylpropanoid pathway, leading to production of phenolic compounds, including phytoalexins. The transient activation of this gene by *Trichoderma* could contribute to the accumulation of phytoalexins, leading further to a better defense of the plants against *Psl* infection.

Hydroperoxide-lyase (HPL) is another enzyme in the octadecanoic pathway. It utilizes some of the LOX products as its substrates, shifting them toward production of antimicrobial and wound-related compounds [also called green leaf volatiles (GLVs)] (249). *Trichoderma* induced *hpl* expression in leaves, but expression was much higher when *Psl* infection followed *Trichoderma* inoculation than with *Psl* infection only (249). This is similar to the priming effect described in the past for rhizobacteria-induced ISR (168, 169).

Ethylene response is considered to be downstream of JA response in rhizobacteria-mediated ISR. ETR1 and CTR1 proteins work together to negatively regulate the ethylene response pathway in the absence of ethylene (122). Ethylene binding to the receptor (ETR) downregulates the activity of this complex and results in derepression of the response pathway. In leaves of *Trichoderma* root-inoculated plants, there was a transitory increase in ETR1 expression followed by a reduction to below control levels, and the expression of CTR1 was almost abolished in plants inoculated with both organisms (204). This may enhance ethylene sensitivity in the leaves, leading to higher defense response in subsequent pathogen challenges. Thus, even though ethylene production did not increase after *Trichoderma* inoculation, the increased sensitivity to the ethylene signal could be the key for activation of this pathway. Interestingly, in roots both genes are induced by *Trichoderma*, suggesting ethylene response is inhibited. This local silencing of plant defense response probably enables symbiotic interactions, as has been observed for other symbiotic systems. Downregulation of the PR protein, PRMS, in roots by *Trichoderma* inoculation is in agreement with local silencing of defense to allow fungal growth into the roots (49). *P. indica* also seems to be able to silence root defense mechanisms and probably for the same reason. Shortly after barley root colonization with *P. indica*, defense-related genes were upregulated (188), but three weeks later no induction of defense-related genes was observed (239). However, ethylene signaling is required for *P. indica* colonization of the roots, as *Arabidopsis* mutants in ethylene signaling were less colonized (213).

Further evidence for the induction of JA and ethylene signaling pathways by *Trichoderma* inoculation comes from other studies (133, 191) and from the study of *Trichoderma* elicitors. Engelberth et al. (75) showed that emission of ethylene, JA,

and volatile compounds related to the octadecanoic signaling pathway are induced in lima bean plants treated with the peptaibol alamethicin from *T. viride*. In addition, Viterbo et al. (236) showed that the 18-residue peptaibols-induced expression of phenylalanine ammonia-lyase (*pal*), hydroperoxide lyase (*hpl*), and peroxidase, which are involved in production of antimicrobial compounds, concomitant with a systemic increase in antimicrobial compounds content in the plant. The SM1-ISR was associated with notable induction of JA and GLV-biosynthetic genes (70), whereas genes involved in SAR were not induced. In the *T. asperellum*–cucumber system as well, the PR proteins chitinase, β-1, 3 glucanase, and peroxidase, which are known to be induced by SA, were not induced by *Trichoderma*, suggesting SAR is not involved. However, if leaves were subsequently inoculated with *Psl*, the expression of these PR genes was much higher than if the pathogen or the *Trichoderma* were used singly (204, 249), again exemplifying the priming phenomenon. This increase in enzyme level was also associated with an increase in phenolic glycoside levels; the aglycones of these materials are strongly antibiotic to a wide range of microorganisms, and *Psl* cell numbers in leaves from plants with both organisms were dramatically decreased compared with plants without *Trichoderma* treatment. Thus, the presence of *T. asperellum* primes the systemic resistance system, but the entire pathway is not constantly turned on. In a subsequent pathogen attack, the plant will react more strongly and/or more rapidly and hence will be more resistant. This implies that certain upstream regulatory genes are activated to provide a much more rapid response than would occur in its absence. The priming mechanism has also been reported in rhizobacteria-mediated ISR and by plant inoculation with *Piriformospora indica (168, 169, 213, 239)*. However, it should be noted that in maize plants inoculated with *T. harzianum* strain T22, at least some PR proteins were constitutively turned on in the presence of the fungus even in the absence of any pathogen (201). Thus, priming may

not occur universally in plant-*Trichoderma* interactions. There is a striking similarity between plant response to *Trichoderma* spp. and *P. indica*. Recent studies show that *P. indica* may induce ISR through the JA/ethylene signaling pathway as well. *P. indica* root colonization reduced powdery mildew infection in *Arabidopsis* wild type and NahG mutant (unable to accumulate SA). However, two jasmonate signaling mutants were fully compromised in *P. indica*–mediated powdery mildew resistance even though their root colonization level did not differ from control plants (213). This indicated that systemic resistance response was independent of SA signaling, but required JA signaling for the process. In addition, *P. indica* colonization of barley roots exhibited no induction of SA biosynthesis genes, but enzymes involved in production of JA and ethylene, such as lipoxygenases and ACC oxidase, were induced (188). Priming was also exemplified in the *P. indica*–plant system for some genes (vsp1 and PR17b) (213, 239). The priming effect was even demonstrated for the pathogen-induced alkalinization response (76).

Recently, it was found that although wildtype strains of *Arabidopsis* were protected against *Pythium* seedling blight in the presence of *T. harzianum* T22, no protection was conferred to *npr1* mutants by T22 (F. Mastouri & G.E. Harman, unpublished data). Like T22, *P. indica* requires functional *NPR1* to induce resistance (213).

Transcription analysis of plant interaction with *T. hamatum* failed to detect induction of ISR markers, and only one marker of SAR (PR5) was upregulated (6). Therefore, it may be that some *Trichoderma* species use other mechanisms to induce plant defense.

Perception of the signal and activation of MAPK signaling cascade

The mechanisms by which these pathways are regulated are no doubt controlled by the interaction of the signal molecules from the BCF with particular plant receptor

molecules in the interaction zone, which further activates a MAPK signaling cascade. Proteomic studies have shown that plant interaction with *Trichoderma* results in induction of NBS/leucinerich repeat resistance protein–like proteins (146, 201). These are known to be part of the specificity determinants of plant immune response, and when activated they trigger a cascade of signal transduction, which results in resistance response. In addition, *P. indica* failed to deliver its effects to *Arabidopsis* plants mutated in a gene coding for a leucine-rich repeat protein, and another receptor protein is required for this process (194). Thus, receptor proteins like these detect the fungus and further deliver the signal of perception.

One plant MAPK protein that is essential to signal transduction in the *T. asperellum*–cucumber system has been identified (199). This protein has been named *Trichoderma*induced MAPK (TIPK). The gene is homologous to *WIPK*, *MPK3*, and *MPK3a* (from tobacco, *Arabidopsis*, and parsley, respectively). *TIPK* is induced by *Trichoderma* in the roots and leaves of cucumber plants. *TIPK* is also activated by pathogen challenge, but its expression was primed when plants were inoculated with *Trichoderma* prior to the pathogen challenge. A unique attenuated virus vector, zucchini yellow mosaic virus (ZYMV-AGII) was used to overexpress TIPK protein and antisense RNA. Plants overexpressing *TIPK* were more resistant to pathogenic bacterial attack than control plants, even in the absence of *T. asperellum* preinoculation. Conversely, plants expressing *TIPK*-antisense revealed increased sensitivity to pathogen attack. Moreover, *Trichoderma* preinoculation could not protect these antisense plants against subsequent pathogen attack. These results demonstrate that *T. asperellum* exerts its protective effect on plants through activation of the *TIPK* gene. Application of JA or SA or inhibitors of JA and ethylene revealed that *TIPK* operates upstream to the JA/ethylene signaling pathways. Using similar

systems, it will be possible to discover regulatory proteins that act earlier in the system and link between *Trichoderma* elicitors interacting with plants receptors to the plant defense pathways activated.

Interestingly, the signal for growth requires *mpk6* because *Arabidopsis mpk6* mutants showed no growth enhancement in response to *Piriformospora indica* inoculation (223). However, the signal for defense response post *Trichoderma* inoculation is delivered through the *mpk3* homolog (199). Whether these two signaling pathways are destined for different plant responses or are different pathways activated by different fungi needs to be determined.

Induction of MAPK signaling cascade is known to induce regulatory proteins. Indeed, many proteomic and transcriptomic studies demonstrate systemic upregulation of regulatory genes and proteins, including RNA binding proteins, elongation factors, GTP binding proteins, and transcription factors (6, 24, 191, 201).

Systemic induction of defense related genes and proteins

Using proteomic analysis of maize plants inoculated with *T. harzianum* T22, a total of 205 differentially expressed spots, over both roots and shoots, were identified with more differences in the shoots than in the roots, even though T22 is present on roots (201). Many proteins of defense/stress-related functions were upregulated. Stress response enzymes such as oxalate oxidase and superoxide dismutase (SOD) were upregulated in roots. In shoots, methionine synthases is highly upregulated. Methionine synthase forms methionine that can further be transformed into S-adenosyl-L-methionine, the precursor of ethylene, which provides additional evidence that ethylene-regulated systems are important in the plant-*Trichoderma* interaction. Other proteins that were upregulated in shoots include glutathione-S-transferase and

glutathione-dependent formaldehyde dehydrogenase (FALDH), which act as detoxifying enzymes; peroxidase, a scavenging enzyme controlling the amount of damage resulting from the oxidative burst; heat shock proteins, which are also a known stress protein; oxalate oxidase, which was found to be involved in stress and defense responses and is probably involved in producing the oxidative burst of hydrogen peroxide; and others (24, 191, 201). Several PR proteins were also upregulated (146, 248).

Six-day-old maize seedlings grown from *T. harzianum* T22–treated seeds had elevated levels of proteins and increased activity levels of chitinase and β-1, 3 glucanase in both shoots and roots. Mostly, these activities were increased further when plants were coinfected with *Pythium ultimum* (109). Not only was total chitinase activity increased but also specific chitinase isozymes were specifically affected by root-inoculation with *Trichoderma* (200). Comparison of the interaction between plants and *Trichoderma* and plant-*Trichoderma*-pathogen indicated the activation of specific response to the biocontrol agent (146).

In T22-inoculated maize plants infected with *P. ultimum*, isoflavone reductase, which is involved in phytoalexin production, was also upregulated (49). The observed induction of phytoalexin accumulation in cucumber and sunflower (137, 249) and the upregulation of proteins involved in the process in maize (49, 201) indicate that this is a common response of defense induction by BCF.

A cell wall biosynthesis enzyme was upregulated in roots, which is not surprising considering that cell wall is being deposited at the site of *Trichoderma* inoculation. In shoots, cell wall metabolism is also activated: Sucrose synthase, UDP-Glc dehydrogenase, and UDPglucuronate decarboxylase were upregulated, and all are part

of the same metabolic pathway for production of cell wall material (hemicelluloses). This may benefit plant resistance by strengthening physical barriers in the shoots (201). Other cell wall metabolism–related proteins were differentially regulated by *Trichoderma* inoculation. Genes encoding extensinlike proteins were upregulated in tomato plants (6) but downregulated in cacao seedlings by several *Trichoderma* isolates (24). Extensin is also involved during rhizobia-mediated ISR. These proteins are involved in cell wall changes, and their metabolism is part of plant mechanism for defense control.

In addition, enzymes that provide protection against oxidative stresses were also upregulated, including glutathione reductase and glutathione S-transferase (6, 19, 24, 201). As noted below, protection against oxidative stress is important in the abilities of BCF to reduce abiotic stress and also to ameliorate the destructive actions of plant pathogenic fungi (131).

Altogether, a whole array of stress- and defense-related proteins are upregulated or primed in plant shoots post *Trichoderma* inoculation of the roots, thus rendering plants to be more resistance to subsequent pathogen attack. Most expression studies of the *P. indica*–plant interaction focus on root response. However, systemic induction of a few defense-related genes/proteins was shown(213, 239), suggesting a similar mechanism.

BCF induced resistance to biotic stress

Recently, several BCF, as well as some plant growth–promoting rhizobacteria (PGPR), have been shown to efficiently help plants overcome abiotic stresses, such as salinity and drought, in both field crops and trees (2, 19, 27, 154, 198, 237, 251). Among the most important stress factors in the field is water deficit. *T. harzianum* added as seed treatment (tomatoes) or as a soil treatment (*Arabidopsis*) largely

improved the germination at osmotic potentials of up to 0.3 MPa (F. Mastouri, T. Björkman, G. Harman, unpublished data). Plants grown from these *Trichoderma* treatments are much more resistant to water deficit conditions. Effects are quite large and probably account for at least a substantial part of the increase in growth of *Trichoderma*-treated versus untreated plants in the field. The ability of maize plants grown from seeds treated with *T. harzianum* to resist water deficit has been demonstrated in the field, and the enhanced deep rooting clearly contributes (98). Moreover, in *Trichoderma*-inoculated cacao seedlings, drought-induced changes such as stomatal closure and reduction of net photosynthesis were delayed under drought compared with noninoculated plants, allowing plants to continue growing (19). In maize, it has been shown that in addition to induction of carbohydrate metabolism and photosynthesis-related proteins, the starch content of the leaves was higher in *Trichoderma*-inoculated plants (201). This could be beneficial for plants under drought, especially if stress is prolonged enough to result in carbon starvation due to prolonged stomatal closure. Water deficit stress induces changes in photosynthetic efficiency, and *T. harzianum* alleviates these effects, described below. Similarly, *P. indica* inoculation of *Arabidopsis* increased drought tolerance(198). Inoculated plants exposed to drought continued to grow, whereas in uncolonized controls, growth was inhibited. Inoculated plants had higher chlorophyll content and higher photosynthetic efficiency under drought-stress.

The ability of BCF to alleviate salt-stress damage to plants was also tested. Growth of most plant species is inhibited by salinity. Salinity affects plants via alterations of water relations in the tissue, disturbances of ion balance, and secondary-induced stresses such as oxidative stress (158). Fresh weight of squash plants was significantly higher in T22- inoculated plants than controls under salinity(251). Similarly, *P. indica*

inoculation abolished the leaf chlorosis and reduced growth that was observed in noninoculated plants under salinity (27, 237). In addition, the rate of metabolic activity increased in leaves of *P. indica*–inoculated plants after salt treatment, suggesting that BCF overcompensate the salt-induced inhibition of leaf metabolic activity (27).

Trichoderma also increased potassium content of plants (250, 251). Salt stress is well known to reduce potassium uptake, and in several systems increasing potassium uptake ameliorated salt-induced damage. Potassium serves as a compatible solute and hence is important to osmotic adaptation. It is also important in stomatal closure control. Therefore, increased potassium uptake can improve a plant's tolerance to water or salt stress–induced osmotic stress. In general, *Trichoderma* can alter the nutritional status of plants (154, 250). Given that salinity negatively affects the plant nutritional status (160), it could be that *Trichoderma* treatment can ameliorate the salt stress–induced growth inhibition through affecting the plant nutritional status. Salinity also reduces calcium content in plants (59, 160), and *Trichoderma* inoculation increased calcium content under salinity compared with nonsaline conditions (251).

A number of other stresses are also alleviated*T. harzianum* has recently been shown to improve resistance to heat and cold (seedlings of tomato were imbibed at 25ºC for 1 day, then exposed to either 10ºC or 35 ºC, and then returned to 25ºC). Seedlings were much less damaged by the temperature extremes in the presence of *T. harzianum.* (F.Mastouri, T. Björkman, G. Harman, unpublished data). Similarly, maize plants with *Trichoderma*-colonized roots were 70% larger at all durations of cold treatment (33).

Trichoderma can greatly induce tree growth in soil that has been used for disposal of building material and sewage sludge (2). After 12 weeks growth, willow saplings grown with T22 in the contaminated soil produced 39% more dry weight biomass and

were 16% taller than the noninoculated controls. In addition, plants inoculated with *Trichoderma* are more tolerant to pathogen attack under salinity stress (154).

T22 provides benefits to seeds under stresses. Seed germination is the first stage of plant growth that must perform well. It is very important to note that effects that occur early in the life of the plant continue throughout the life of at least annual plants (92, 98). Seeds exposed to abiotic stresses, including osmotic-,salt-, heat-, and cold stresses, in the presence of T22 have much higher percentages of germination and improved seedling vigor (Mastouri F, Björkman T, Harman G). Tomato seed lots with reduced vigor caused by various aging regimes exhibit higher percentages of germination and improved seedling vigor compared with nontreated seeds (Mastouri F, Björkman T, Harman G). Conidia of T22 added as a seed treatment benefits the seed by an increase in phase III imbibition (cell elongation, followed by radicle protrusion). The seed response is rapid and appears to begin before the fungus penetrates into the living portions of the seed.

A volatile elicitor from T22 that enhances tomato seedling growth at 400 $pg.L^{-1}$ has been identified. These data suggest that T22 produce volatile elicitors that enhance plant performance even at a distance. Other volatiles such as 6-n-pentyl-6H-pyran-2-one that induce plant growth have been isolated from different *Trichoderma* strains(232).

Alleviation of damage by reactive oxygen species

Under severe stress, ROS production can exceed the scavenging capacity and accumulate to levels that can damage cell components, e.g., via lipid peroxidation (151). It seems that endophytic fungi on roots can modulate the damaging levels of ROS, thus symptoms of biotic and abiotic stress can be limited. Roots and leaves of *P.*

indica–inoculated plants showed increased levels of antioxidant compounds and antioxidative enzymes and reduced levels of hydrogen peroxide (27, 187, 237). *Trichoderma* spp. also enhances protection against ROS possibly by increasing ROS scavenging abilities. Proteomics of roots inoculated with *Trichoderma* identified increased levels of SOD as well as increased levels of peroxidase, glutathione-reductase and glutathione-S-transferase (GST), and other detoxifying enzymes in leaves(201). A peroxidase gene was also primed in pathogen-infected cucumber plants inoculated with *Trichoderma* (204). Seeds that were subjected to oxidative stress had much reduced vigor, but subsequent treatment with *Trichoderma*-T22 restored vigor (33). In a recent study, treating seeds of tomato with *T. harzianum* T22 enhanced germination percentage under osmotic stress (Mastouri F, Björkman T, Harman G). We found an increase in lipid peroxide content in young seedlings with increase in the water potential of media, whereas T22-treated seedlings had significantly less lipid peroxide than untreated seedlings (Mastouri F, Björkman T, Harman G). Similarly, root colonization by *P. indica* attenuated the salt-induced ROS damage (27). Altogether this suggests that BCF alleviates stress damage through controlling ROS damage.

Involved in plant response to abiotic stress

Various proteomic and transcriptomic studies published also provide clues for BCF ability to induce abiotic stress tolerance. *Trichoderma* induce various proteins/genes involved in stress response in different plants, including antioxidative response such as peroxidase and H2O2 producers such as oxalate-oxidase and glucose-oxidase (24, 191, 201). FALDH and GST were upregulated (24, 201). Among their many activities, they have a broad role in protecting cells from oxidative injuries by detoxifying compounds that would otherwise damage plant cells. The heat shock protein group of

chaperones was also upregulated (191, 201). Interestingly, ornithine decarboxylase, a primary control point in polyamine biosynthesis, was upregulated in cacao seedlings by several *Trichoderma* isolates (19). Polyamines have been associated with abiotic stress, and modulation of their biosynthetic pathway confers tolerance to drought or salt stress (45, 136). In addition, tonoplast intrinsic protein, a member of the major intrinsic proteins (MIPs), was downregulated in cacao seedlings by several *Trichoderma* strains (19). Members of the MIP superfamily in plants function as membrane channels that selectively transport water out and between cells, and their expression declines in response to drought (125, 210). The repression of MIP expression may reduce membrane water permeability and encourage water conservation during periods of drought (210). Moreover, changes in droughtinduced gene expression in leaves were delayed by three days in *Trichoderma*-inoculated cacao seedlings(19). This may be the result of increased water content in these seedlings, which may have caused a delay in drought response. Additionally, *Trichoderma* induced osmotin-like, salt-induced proteins in tomato plants (6). Drought stress–related genes were upregulated sooner and to a higher extent in *P. indica*–inoculated plants (197). These included marker genes involved in drought stress tolerance, *RD29A* and *ERD1*, and other drought stress–related genes encoding for proteins involved in signaling, protein degradation, and control of gene expression. Also included in those genes is a histone acetyl transferase (HAT), which may be involved in chromatin histone acetylation. Chromatin acetylation serves as a general transcriptional regulation mechanism; hence it may imply that BCF can control gene expression more generally by regulating factors involved in histone acetylation, but this needs to be studied further. Overall, modulation of different biochemical pathways involved in adaptation of plants to abiotic stress by BCF inoculation can result in the observed enhancement of abiotic tolerance in different plants.

Plant growth enhancement by BCF inoculation

It has long been known that BCF can enhance plant growth. *Trichoderma* and *Sebacinales* species inoculation induce root and shoot growth (28, 92, 98, 103, 109, 167, 170). *Trichoderma* even promoted growth of trees (2, 19). BCF also increase percentages of germination and rates of germination of seeds (28, 33)(10, 14, 19, Mastouri F, Björkman T, Harman G). The application of *Trichoderma* led to an increase in drymatter content, starch, total and soluble sugars, and a reduction in sugar content in leaves of different plants (2, 137, 201). *P. indica* can promote adventitious root formation in cuttings and may thus be a good candidate for biological hardening of micropropagated plantlets (71, 205). More importantly, the effect of BCF on plant growth has a long duration and even last for the entire life of annual plants (28, 98, 103, 237). Other nonpathogenic root colonizing fungi also have similar abilities (139).

It is possible that BCF affect growth by counteracting deleterious root-associated microflora. However, *Trichoderma* and *Sebacinales* species were shown to induce growth under sterilized and nonsterilized conditions (109, 139, 167, 205, 250), suggesting a direct mechanism through plant response.

The activation of direct defense reactions has a metabolic cost that reduces plant fitness (67, 112, 113, 220). However, rather than reducing growth, BCF typically either have no effect or substantially increase plant growth. Van Hulten et al. (228) have shown that priming has a smaller effect on fitness than directly induced defense. Thus, priming reduces the energetic costs of plant readiness status to resist pathogens. In other cases, defense mechanisms and PR expression are induced (191, 200, 201), yet growth is not compromised, probably because of increased energy supply to cover the energetic cost (200, 201). However, in some systems the metabolic costs cannot be

covered by the growth enhancement, as has been demonstrated for *Sebacina vermifera* (28).

This greater energy supply must ultimately come from photosynthesis, and probably needs to be accompanied by greater respiratory rates (201). This indeed appears to occur. Recent progress in elucidating the direct effect of *Trichoderma* on plant growth was obtained from a proteomic study of the T22-maize system, which demonstrates highly reproducible growth promotion (201). Of the 205 differentially expressed proteins in the presence of *Trichoderma*, the most commonly affected were those involved in carbohydrate metabolism, especially those in the glycolytic, tricarbixylic acid (TCA) or respiratory pathways, and most of them were upregulated in the shoots (200, 201). Some of the carbohydrate- and respiratory-related proteins were also found to be upregulated in studies involving other *Trichoderma* species (44, 191). In addition, several photosynthesis-related proteins were upregulated in plants by the interaction with *Trichoderma*. Maize plants inoculated with T22 have higher leaf greenness than noninoculated plants (98). Together, this suggests that *Trichoderma* can increase photosynthetic capacity of the plants.

Improved photosynthetic abilities

In addition to increased levels of photosynthetic apparatus, photosynthesis can also be improved by higher photosynthetic efficiency.When measuring fluorescence kinetics and using Fvariable/Fmaximum ratio (F_v/F_m) as a measure of photosynthetic efficiency (32), no difference was found between control- and *P. indica*–inoculated plants under nonstress conditions(167), nor was there any difference in chlorophyll content. However, fast chlorophyll fluorescence kinetics (O-J-I-P) used to analyze photosynthetic efficiency demonstrated that the yield for electron flow was

substantially increased by root colonization with the fungus and that electron transport per trapping center was strongly enhanced. This improvement in photosynthetic efficiency was strongly related to plant height and root colonization by *Piriformospora indica* (171).

Transferring *Arabidopsis* seedlings adapted to low light to higher light conditions severely damaged seedling growth and foliages turned red. However, no obvious damage was observed in plants inoculated with *Trichoderma*-T22. The F_v/F_m of control plants was reduced to 0.45, but this value for T22-treated plants was 0.78 (F. Mastouri & G.E. Harman, unpublished data). The value for unstressed *Arabidopsis* leaves is approximately 0.80 (198). Thus, the photosystem in *Arabidopsis* in the presence of T22 under light stress was near optimal (Mastouri F, Björkman T, Harman G).

Rapid chlorophyll fluorescence transients analysis is a nondestructive and sensitive method that can detect water deficit stress before irreversible wilting and damage occur (164), whereas the F_v/F_m parameter decreases only at approximately the time that plants suffer irreversible water deficit stress (243). . Oukarroum et al. (164). identified a performance index (PI) based on the measured parameters that accurately calculated the intrinsic resistance to water deficit and that differentiated between barley cultivars varying in resistance to water deficit. Using this method, we showed that *T. harzianum* substantially reduced effects of water deficit even after two weeks of withholding irrigation, when plants were at or approaching the permanent wilting point. Even after just one week, the PI was significantly different: In plants grown from seeds treated with T22, PI = 0.601±0.071 (mean±SE), and in plants grown from untreated seeds, PI = 508 ± 0.067, whereas other parameters such as F_v/F_m were not changed at the early stages of water deficit stress. These results demonstrate that

Trichoderma strains reduce effects on photosynthetic systems as they increase water deficit tolerance in a manner similar to that demonstrated by differentially resistant barley lines (164).

Altogether, BCF enhance plant growth at least in part because respiratory systems and carbohydrate metabolism are upregulated and thereby increase energy and sugar supply to the growth of the plant. This is probably maintained by the increase in photosynthesis. In a study with tomato plants inoculated with *T. hamatum*, the expression of stress-, cell wall–, and RNA metabolism–related genes was also upregulated, demonstrating similarities of plant responses to *T. harzianum* (6). However, in this system carbohydrate metabolism–related genes were not upregulated, and no positive growth response was recorded. This strengthens our suggestion that there is a direct connection between the ability of *Trichoderma* to induce energy metabolism and its ability to induce growth response. A summary of plant responses is depicted in Figure 1.

Effect on root development and performance

Inoculation of plants roots by *Trichoderma* or *Sebacinales* species results in changes of root development. *Trichoderma*-inoculated roots are deeper and more robust (98). Main and secondary roots of maize increased in size, and the area of the root hair was greater with *Trichoderma*-T22 inoculation (109). *Piriformospora indica* also induced root developmental changes. Promotion of root growth and increased length of root hairs were detectable even before notable root colonization (167). Root branching can improve soil exploitation and hence result in plant growth promotion. An effector hydophobin-like protein from T22 has been identified that mimics the effect of the fungus (180). *Arabidopsis* root colonization by *P. indica* resulted in a stunted but

highly branched root system, which is probably mediated by low amounts of auxins produced by *Piriformospora indica* (224). Several auxin-like secondary metabolites produced by *Trichoderma* strains were able to induce plant growth and are required for development of lateral roots in *Arabidopsis* (57, 232). However, a recent study implicates cytokinins in plant growth promotion (224). *P. indica* induces relatively high levels of cytokinins, and the cytokinin levels are higher in colonized roots compared with uncolonized controls. Although root colonization was not affected in cytokinin biosynthesis or receptor mutants, no growth response was recorded in mutants possessing decreased levels of *trans*-Zeatin cytokinins. This indicated that cytokinins are required for the plant growth response but not the root interaction with the fungus (224).

Although *P. indica* infects mainly thedifferentiation zone of the roots, from which it can spread, and causes localized cell death, *Trichoderma* spp. have the abilities to rapidly colonize seeds and immediately confer benefits to seeds and seedlings even before radicle protrusion occurs (Mastouri F, Björkman T, Harman G). This allows *Trichoderma* spp. to be used widely as seed treatments, which is a very cost-effective method of application. Given that *Trichoderma* strains can grow with and keep up with the developing root system, long-term protection (96-98) occurs even though the initial application rate is only one gram or less per hectare.

Another component of growth induction could be due to increase in nutrient uptake. *Trichoderma* spp. have significant abilities to solubilize a range of plant nutrients, such as phosphorus and micronutrients including iron, copper, zinc, and manganese, thus rendering them available for plants (10). In addition, even in hydroponics with fully soluble and available nutrients, the presence of *T. asperellum* on cucumber roots increased the uptake of a similar range of plant nutrients(250). *P. indica* can also

mediate solubilization and translocation of minerals to plants (84, 167, 205), although there are some contradicting results (28). When interaction of plants with *P. indica* was underbalanced, nutrient-supply, no-shoot growth enhancement was observed, whereas under low nutrient or poor soil, *P. indica* induced plant growth (167, 193, 207). It could be that the balanced nutrient supply obscured the advantage conferred by root branching, and hence growth can be promoted by *P. indica* under suboptimal conditions. However, even under near-optimal conditions, *Trichoderma* seed treatment of maize sometimes gives improved yields (95, 98, 109). Thus, BCF can solubilize plant nutrients (indirect effect) and also induce plants to uptake more nutrients (direct effect). There is no doubt that the increased root development associated with colonization of plant roots by BCF contributes to this and other benefits to plants.BCF treatments therefore have the potential to improve overall crop yield and may be particularly important in suboptimal field conditions.

Enhanced nitrogen use efficiency

BCF increase nitrogen use efficiency (NUE) in plants (93, 98, 171, 197). This effect was first noticed with *T. harzianum* T22 in maize field trials in the late 1990s (98). Plants grown under conditions of low soil nitrogen from seeds treated with T22 were larger and darker green (98). Plants generally respond to increasing nitrogen fertilizer levels with increased yield and growth up to a point when increasing nitrogen fertilizer no longer increases yields. In the presence of T22, this yield plateau was reached with 40–50% less nitrogen fertilizer than in its absence (93, 98). This increase in plant NUE is a long-term effect and can be induced by a seed treatment whose effect persists for the productive lifetime of the crop. Although, in maize, a few genotypes respond negatively to *Trichoderma* (101), seed treatment of wheat with this fungus is highly effective, nearly always giving improved yields (92). This ability is being exploited in

the United States, and approximately 0.3 million hectares of wheat are being planted with seeds treated with *T. harzianum* strain T22. Our new understanding of the mechanisms of action of *Trichoderma* strains, and their broad capabilities, permitted improved screening protocols, and new strains were selected and compared forNUEimprovements with the former strain *T. harzianum* T22. The new strains *T. harzianum* RR17Bc and *T. atroviride* WW10TC4 were selected because they had better growth induction capabilities than T22, and *T. harzianum* F11Bab was selected because it was improved over T22 in inducing systemic disease resistance (92). Nitrogen rate ranging trials were conducted in large pots in the greenhouse, which are the only way to fully define the yield/nitrogen uptake effects of added *Trichoderma* (F.Mastouri&G.E. Harman, unpublished data). Nitrogen content increased in *Trichoderma*-treated plants, but moreover, correlation analyses over several strains and nitrogen levels revealed that nearly all of the yield variation could be explained by the nitrogen variation levels in plants. Thus, the strains improve yields by increasing NUE. Strain WW10TC4 of *T. atroviride* appeared to be the most effective. Moreover, both *Trichoderma* and *P. indica* induce expression of nitrate reductase in plants (19, 197) that convert nitrate to ammonium ions (the required form for nitrogen metabolism) (197), and hence they may be involved in nitrogen accumulation through the symbiotic association. NUE in *P. indica*–colonized plants is associated with a marked increase in nitrate reductase.

Similarities of beneficial effects of distantly related endophytic plant microbes: an example of convergent evolution?

Throughout this review, we have pointed out the similarities in effects and mechanisms including *Trichoderma* spp., which are ascomycetous fungi; *P. indica*, which are basidiomycetous fungi; and various PGRPs, which are Eubacteriales. These

effects and mechanisms are summarized in Table 1. Although we have until recently considered these and similar microorganisms as BCFs, it is clear that their benefits are much greater than just the control of plant pathogens. These organisms are very distant from one another and represent dissimilar genetic lineages

Each organism mediates systemic resistance, mostly by ISR, although other mechanisms may also be involved (it probably depends on which elicitors are involved). Where it is known, they also induce a considerable amount of tolerance to plant abiotic stresses, which is a recently discovered advantage of these organisms. Further, some increase NUE in plants, which is perhaps the most significant advantage of these organisms because it can reduce environmental pollution and enhance food security, especially in developing countries. Nitrogen is expensive and thus is limited in less wealthy regions of the world, and thereby food production is reduced.

The organisms that appear in Table 1 have demonstrated abilities to reduce accumulation of damaging levels of ROS during plant stress. This is probably associated with an increase of photosynthetic efficiency, especially during stressful conditions.

T. harzianum strain T22 appears to have an additional benefit. It alleviates physiological stresses in seeds caused by poor seed quality (14, Mastouri F, Björkman T, Harman G). These effects happen very quickly; when seeds are treated with the conidia of the organism, the conidia germinate only 18–20 h after imbibition begins and then must traverse the seed coat before reaching the living portions of the seeds. However, effects are noted by the time radicle protrusion occurs (48–96 h after imbibition begins), which means that the effects of the fungus had to occur earlier,

Table 4. 1. Comparison of three distantly related root-colonizing microbes (endophytic plant symbionts) on plant growth and biochemical mechanisms.

Plant effect or mechanism	*Trichoderma* spp.	*Piriformaspora indica*	Plant growth promoting rhizobacteria	References
Internal root colonization	+	+	+	50, 64, 90
Improved plant shoot and root growth	+	+	+	46, 64, 125
Systemic resistance to disease	+	+	+	50, 63, 126
Induced resistance to abiotic stresses	+	+	+	52, 63, 125
Enhanced root development/adventitious root formation	+	+	/+	34, 48
Enhanced general uptake or solubilization of plant nutrients	+		+	4, 40
Increased plant nitrogen use efficiency	+	+	-/?	46, 100
Enhancement of performance of physiologically impaired seeds	+	-	-	76
Mechanisms				
Induced systemic resistance	+ *	+ *	+*	63, 64, 104,106, 111
Amelioration of oxidative damage induced by stress	+	+	+	40, 65, 76
Enhanced activity of nitrogen reductase in plants	?	+	?	100
Enhanced photosynthetic capability and/or efficiency especially in plants under stress	+	+	+	40, 46, 87, 105

* Other mechanisms may also be induced

probably in stage 2 of germination, approximately 24–36 h after the start of imbibitions. These data suggest the benefit happens before the living portions of the seeds are colonized, which suggests the involvement of highly effective licitors.Weare currently investigating volatile *Trichoderma* metabolites for this role (Mastouri F, Björkman T, Harman G).

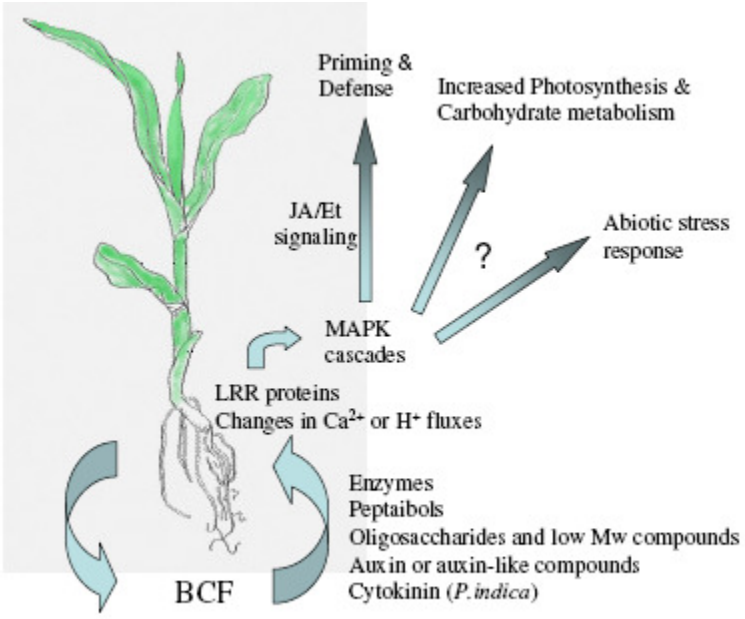

Figure 4. 1. BCF-plant interaction
BCF grows to interact with the roots. By forming this interaction the BCF and the plant exchange signals. BCF releases elicitors into the zone of chemical communication (Both outside and inside root tissue) and activates a MAPK cascade in the plant. The JA/Ethylene signaling pathway is being activated which results in priming and /or increase of plant defense genes which ultimately increase plant resistance to pathogens. In addition, increase of carbohydrate metabolism and photosynthesis change the source-sink relationship resulting in more energy and carbon source to the growing plant leading to the observed enhanced growth response. At least for *Trichoderma* we know that there are strains that induce defense but not growth and *vise versa* suggesting the signaling pathways leading to these plant responses are different. Whether these signaling pathways also differ from the one leading to abiotic stress responses needs to be determined.

It is unlikely that these diverse organisms trigger their beneficial mechanisms in the same way. Indeed, given the range of elicitors produced by *Trichoderma* spp., there probably are different mechanisms/triggering molecules produced by these fungi. Nonetheless, similar responses occur in a diverse range of plants, and the basic mechanisms seem to be similar. The abilities of these fungi to elicit similar end responses may suggest that these mechanisms are the ones that are most likely to benefit plants across a range of species and climatic conditions.

Of course, the organisms discussed here are those whose lifestyles are benefited greatly from large numbers of healthy roots. This provides nutrients and protection of these fungi against competitors. Certainly with *Trichoderma* spp., greater numbers of the fungi are found when large numbers of healthy roots are present (109). This is further evidence for the symbiotic nature of the relationship between these organisms and plants. It is probable that the elicitors and specifics of the specific elicitation of responses differ between the microbes, and this ought to be a fruitful area to consider for the nongenetic improvement of plant performance. The authors are working to provide widescale systems for use with *Trichoderma* spp. to improve plant productivity and plant health (for resistance to biotic and abiotic stresses) and to use NUE to increase food and fiber production, while reducing environmental damage.

Conclusion

Although general mechanisms regarding plant response to BCF inoculation can be derived from the studies described above, it is important to remember that natural variations do occur. For example, some *Trichoderma* species induce growth in addition to ISR (92), whereas others do not (5). Biocontrol *Trichoderma* strains were also reported that do not induce resistance or enhance growth despite their endophytic

abilities (64). Bailey et al. (24) have demonstrated that plant gene expression profiles depend on the *Trichoderma* isolate colonizing the plant. The responses depend not only on the BCF used but also on the plant species or cultivar. *Trichoderma*-treated maize has an average yield increase of approximately 5%, but there are significant varietal differences, with some maize lines giving neutral or even negative growth responses (101). However, yield responses of T22-treated wheat appears to be extremely robust in the field (110). Different *Arabidopsis* ecotypes also respond differently to *P. indica* or *Trichoderma* spp.. Once the specific control mechanisms of the BCF-plant interaction are known, then very specific genetic lines that have favorable outcomes can be readily identified and used. Moreover, knowledge of specific critical gene products that are associated with favorable outcomes will permit rapid assays of the expression of critical proteins or genes even on a field scale. This will provide a major management tool that will afford reliable assessment of the interaction. We believe that the abilities of these fungi to (*a*) induce resistance to biotic stresses such as disease and abiotic stresses such as drought and salinity and (*b*) increase NUE make them extremely useful tools with which to increase plant productivity, improve food security, and improve the environment. Specifically, these fungi's ability to produce NO compounds from unused fertilizer can reduce nitrogen fertilizer application, thereby reducing nitrate pollution of waterways and air pollution(89, 128, 140).

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