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Physiological and Molecular Plant Pathology 65 (2004) 49–56

PMPP

www.elsevier.com/locate/pmpp

The mycotoxins beauvericin and T-2 induce cell death and alteration to the ascorbate metabolism in tomato protoplasts

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Accepted 23 July 2004

Abstract

The phytotoxicity of the mycotoxins from *Fusarium* spp. beauvericin and T-2 toward tomato protoplasts was evaluated. Both toxins caused protoplast death, but the phytotoxicity of beauvericin was much more severe in terms of reduction in protoplast viability. The possible involvement of the ascorbate system in the response induced by the two mycotoxins was also studied. Tomato protoplast death was preceded by an imbalance of the ascorbate system, which was diversely affected by the two mycotoxins. Beauvericin caused a decrease in the ascorbate level, whereas T-2 induced a significant increase in dehydroascorbate. The changes in the activity of ascorbate peroxidase, the most efficient scavenger of hydrogen peroxide in plant cells present in all cell compartments, were also analysed. Ascorbate peroxidase activity decreased after 3 h of treatment with beauvericin. The activity of dehydroascorbate reductase, the enzyme involved in ascorbate regeneration from its oxidized form, also increased within the first hour of treatment. T-2 treatment did not induce significant changes in either ascorbate peroxidase or dehydroascorbate reductase activities. The possible role of the ascorbate system in tomato protoplast death induced by treatment with beauvericin and T-2 mycotoxins is discussed.

Keywords: Ascorbate; Cell death; Dehydroascorbate; *Licopersicon esculentum*; Mycotoxins; Phytotoxicity; Protoplast viability

1. Introduction

Fungal pathogens produce a large number of secondary metabolites which are toxic for plants and often contribute to disease. T-2 toxin (T-2) and beauvericin (BEA) are mycotoxins produced by several species of the *Fusarium* genus, a common contaminant of cereals [1,2]. BEA is a cyclic hexadepsipeptide with insecticidal [3], antibiotic [4], apoptotic [5], and cholesterol acyltransferase-inhibitory properties [6]. The toxic effects of BEA have been studied by biological assays both in various species of invertebrates [4,7] and, in vitro, in mammalian cell lines [8], where it is effective at micromolar concentrations. Although recent studies have shown that BEA is produced by various phytopathogenic *Fusarium* species [2,9], suggesting that the toxin may play an important role in the etiology of plant diseases, limited information exists on the toxigenicity of BEA against plant systems. Preliminary studies reported that BEA was highly toxic to melon protoplasts compared with fusaric acid and fumonisin B_1 [10].

Differently from BEA, the toxicity of T-2 toward humans, animals, and plants systems has been well studied. In particular, T-2 is a potent eukaryotic protein synthesis inhibitor [1] and, at least in animals, induces the production of free radicals which are responsible for lipid peroxidation and alteration of the structure of cell membranes [11,12]. T-2 is found as a natural contaminant of various plants [13] and shows high phytotoxicity [14]. Among 16 different metabolites sharing trichothecene structure, the toxicity of which has been studied on plant systems, T-2 showed the highest relative toxicity [15].

Abbreviations: APX, ascorbate peroxidase; ASC, ascorbate; BEA, beauvericin; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; ROS, reactive oxygen species.

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doi:10.1016/j.pmpp.2004.07.006

In plants there are many potential sources of reactive oxygen species (ROS) [16]. It is well known that toxic ROS levels cause lipid peroxidation so damaging nucleic acids and proteins to the point of inducing cellular dysfunction and/or death. In a variety of plant–pathogen interactions microbial phytotoxins induce the production of ROS [17] and it has been suggested that the fate of the infection mainly depends on the capability of the plant to modulate such a ROS increase opportunely [18].

In plant cells ROS detoxification is carried out by a network of reactions involving enzymes and metabolites with redox properties. Ascorbic acid (ASC) and its related enzymes play a key role in the detoxification of ROS [19]. ASC can either directly react by reducing oxygen free radicals or be involved, as a substrate of ascorbate peroxidase (APX; EC 1.11.1.11), in the scavenging of hydrogen peroxide [19]. In higher plants APX is one of the main detoxifying enzymes. It has been studied in many species of Angiosperms and Gymnosperms [20] and is present in almost all cellular compartments [21]. Among the different APX isoenzymes, the cytosolic ones seem to be the most involved in the responses against stress. Their expression/activity is increased under several abiotic stresses, even when the major alterations concern the photosynthetic apparatus [22], whereas their suppression seems to be a critical point for the success of programmed cell death in plants, and in the hypersensive reaction [16,23]. Dehydroascorbate reductase (DHAR; EC 1.8.5.1), the enzyme that reconverts dehydroascorbate (DHA), the final oxidized form of ASC, is also involved in the responses against stress [16] and, similarly to APX, is present in all the organelles in which ASC is utilised [24].

The aim of the study described here was to investigate the effects of BEA and T-2 on tomato plants and the involvement of ASC metabolism, in particular the level and redox state of the ASC/DHA redox pair and the activities of the cytosolic APX and DHAR, in the metabolic perturbations induced by the two mycotoxins. When studying the incorporation of complex organic compounds, intact tissues may constitute an unsuitable system owing to the interference of cuticle and cell cohesion that hamper solute transport thus inducing non-homogeneous incorporation [25]. In this respect, protoplasts represent a more suitable system and can be used as a model system to evaluate the phytotoxicity of mycotoxins [10]. For this reason, the effects of the mycotoxin were studied on freshly isolated tomato leaf protoplasts.

2. Materials and methods

2.1. Chemicals

T-2 and BEA toxins were purchased from Sigma (Sigma-Aldrich Chemie, Steinheim, Germany). Since both compounds are poorly soluble in water, a 1.28 mM stock solution in methanol of each was prepared. The stock solution was further diluted to the desired concentration.

2.2. Plant growth and protoplast isolation

Seeds of *Lycopersicon esculentum* L. cv. Marmande were germinated at 23 ± 1 °C and 55–60% relative humidity under white fluorescent light (24 μmol m^{-2} s^{-1}) with a light/dark photoperiod of 14/10 h.

The youngest fully expanded leaves of 28–30-day-old plants were harvested and utilized for protoplast isolation. Prior to the isolation of protoplasts plants were kept in the dark for 24 h. Tomato leaf segments of 2–3 g deprived of the lower epidermis were incubated at room temperature for 30 min in 20 ml of 25 mM Tris–Mes pH 5.5 containing 0.6 M sorbitol, 0.5% BSA and 0.5% $CaCl_2$ (pre-plasmolysis buffer). The incubation medium was then replaced by 20 ml of the same medium supplemented with 1% (w/v) Caylase (Cayla, Toulouse, France), 0.02% (w/v) Pectolyase and 0.2% (w/v) Pectinase (Sigma-Aldrich Chemie, Steinheim, Germany). The digestion proceeded for 4–5 h, at room temperature in the dark. Protoplasts were released by gentle stirring, filtered through nylon cloth (75 μm mesh), and centrifuged at 50*g* for 6 min at 4 °C. The pellet was washed four times with pre-plasmolysis medium adjusted at pH 7.4 and re-suspended to the final concentration of 1×10^5 protoplast ml^{-1}. After isolation, protoplast viability was $85 \pm 2\%$ ($\pm$SE). About 0.2 ml aliquots of the protoplast suspension were utilized for incubation with BEA and T-2 at the concentrations of 50 and 100 μM and for different times. Control protoplast suspensions contained the same amount of MeOH as the treated sample. Protoplast viability was estimated by the method of Kanai and Edwards [26]. Briefly, samples of the incubation with toxins, were taken at intervals and the protoplast suspensions were mixed with an equal volume of 0.25% (w/v) Evans blue. After 10 min protoplasts were examined under a light microscope. Evan's blue dye enhances the differences between live and dead protoplasts since it is excluded by intact protoplasts, while the dye is strongly absorbed by broken protoplasts. The exclusion of dye by intact protoplasts is taken as an estimate of protoplast viability.

Five separate experiments were performed to estimate the levels of toxicity of BEA and T-2. In each experiment replicate samples ($n = 4$) were taken, so that a total of 1000–1200 protoplasts was analyzed in each assay. Results are presented as percent viability.

2.3. Measurement of H_2O_2

Intracellular H_2O_2 production was measured using dihydrorhodamine 123 (DHR123) (Sigma-Aldrich Chemie, Steinheim, Germany) as a probe [27]. The protoplasts were stained for 5 min with 20 μM DHR123 and then viewed under a fluorescence microscope (DMSL, Leica) with an

excitation filter of 450–490 nm and a barrier filter of 510 nm.

To determine the amount of H_2O_2 released in the medium, aliquots of incubated protoplasts were pelletted by centrifugation and the H_2O_2 concentration was measured in the supernatant according to Bellincampi et al. [28].

2.4. Analysis of ascorbate and glutathione pools

Aliquots of incubated protoplasts were pelletted and homogenized in a suitable medium using a conical pestle (motor cordless Kontes). For determinations of ASC, DHA, reduced glutathione (GSH) and oxidized glutathione (GSSG), samples were homogenized in two volumes of cold 5% (w/v) metaphosphoric acid. The homogenate was centrifuged for 15 min at 20,000*g*. The ASC, DHA, GSH and GSSG contents of the supernatant were measured according to Zhang and Kirkham [29].

2.5. Enzyme assays

For the determination of enzymatic activities samples were homogenized in 50 mM Tris–HCl, pH 7.8 containing 0.3 mM mannitol, 1 mM EDTA, and 0.05% (w/v) cysteine. For GLDH analysis 0.1% (v/v) Triton X-100 was also added. The homogenate was centrifuged for 20 min at 25,000*g*. The supernatant was desalted by dialysis against 50 mM Tris–HCl, pH 7.8, and used for spectrophotometric analysis. All procedures were carried out at 4 °C.

Ascorbate peroxidase (L-ascorbate: hydrogen peroxide oxidoreductase, EC 1.11.1.11) activity was determined following the decrease in absorbance at 265 nm in a reaction mixture containing 50 μM ascorbate, 90 μM hydrogen peroxide, 50 mM phosphate buffer, pH 6.5, and 50 μg protein [30]. The oxidation of ASC non-dependent on H_2O_2 addition was subtracted. Since ASC was absent in the extraction buffer, the measured activity should only have been due to cytosolic ascorbate peroxidase [21].

DHA-reductase (glutathione: dehydroascorbate reductase, EC 1.8.5.1) activity was determined following the increase in absorbance at 265 nm in a reaction mixture containing 1 mM DHA, 1 mM GSH, 100 mM phosphate buffer, pH 6.3, and 50 μg protein [31]. The rate of non-enzymatic DHA reduction was subtracted. Both ascorbate peroxidase and DHA-reductase activities were estimated by assuming a molar absorbance coefficient for ASC of 14,000 M^{-1} cm^{-1} at 265 nm.

Catalase (hydrogen peroxide: hydrogen peroxide oxidoreductase, EC 1.11.1.6) activity was determined according to Beaumont et al. [32] with minor modifications, by following the H_2O_2 dismutation at 240 nm in a reaction mixture containing 0.1 M phosphate buffer, pH 7.0, 18 mM H_2O_2 and 50 μg protein (extinction coefficient 23.5 mM^{-1} cm^{-1}).

GLDH (L-galactono-1,4-lactone: ferricytochrome-*c* oxidoreductase, EC 1.3.2.3) activity was determined according to Ôba et al. [33] with minor modifications, following the reduction of cytochrome *c* at 550 nm. The reaction medium contained 50 mM Tris–HCl buffer, pH 8.0, 60 μM cytochrome *c*, 1 mM sodium azide, 1 mM L-galactono-1,4-lactone (GL), 1 μM FAD and 50 μg protein and utilising an extinction coefficient of 27 mM^{-1} cm^{-1}. Sodium azide was added to inhibit cytochrome oxidase activity thus avoiding underestimation of GLDH. The variation in absorbance not dependent on GL was subtracted.

The protein content was determined according to Bradford [34], using bovine serum albumin as a standard.

2.6. Lipid peroxidation

The level of lipid peroxidation in the protoplasts was measured in terms of malondialdehyde (MDA) content determined by the thiobarbituric acid reaction as described by Zhang and Kirkham [29].

2.7. Statistics

Statistical analysis of the differences between mean values of control and treated protoplasts was performed using the Student's *t* test. Differences at $P < 0.05$ were considered significant.

3. Results

3.1. Time course of protoplast killing

The effects of BEA and T-2 at either 50 or 100 μM on tomato protoplast viability are shown in Fig. 1. These toxin concentrations were chosen for two reasons: (a) similar concentrations are produced by various *Fusarium* species on plant matrices [1,2] and (b) similar concentrations proved to be toxic toward other bioassays [10,14].

Compared to the control, the protoplast viability was 87% after the first hour of incubation with 50 μM BEA, 70% after 2 h and 50% after 3 h. After 6 h protoplast viability was only 9% of that of the control (Fig. 1A).

The treatment with 50 μM T-2 only resulted in a significant reduction in protoplast viability after 6 h (Fig. 1A). A dose–response relationship was shown by increasing the concentration of both toxins to 100 μM (Fig. 1B). At this concentration, the reduction of protoplast viability following treatment with BEA was already statistically significant after 30 min, and that induced by T-2 after 3 h.

Viable protoplasts, both in the control and after different treatments, appeared spherical shaped with well-defined chloroplasts. No alterations of protoplast morphology occurred during the first 30 min of treatment with BEA or during the first hour of treatment with T-2 (data not shown). The toxic action of the two mycotoxins was shown by

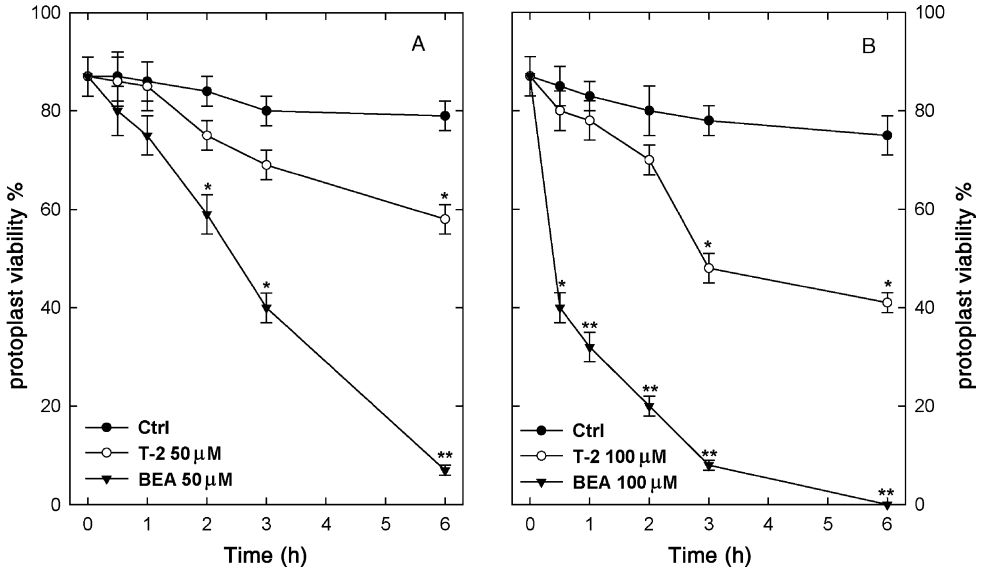

Fig. 1. Effects of BEA or T-2 toxins on protoplast viability. The protoplasts were treated with 50 (A) or 100 µM (B) toxin. Values represent the mean of five experiments ±SE. * and ** indicate values significantly different from the control by Student's *t* test with $P<0.05$ and 0.01, respectively.

the appearance of altered morphology of protoplasts, which resulted in protoplast death. Dead protoplasts showed both cytoplasm and chloroplast disruption with increasing damage to the plasma membrane until complete lysis (data not shown).

3.2. H_2O_2 production in treated protoplasts

To ascertain whether BEA and T-2 induced ROS generation, the H_2O_2 production in control and treated protoplasts was monitored. The production of extracellular H_2O_2 induced by 50 µM BEA and T-2 was investigated at different times of treatment (Fig. 2). The level of H_2O_2 increased both in BEA and T-2 with respect to the control

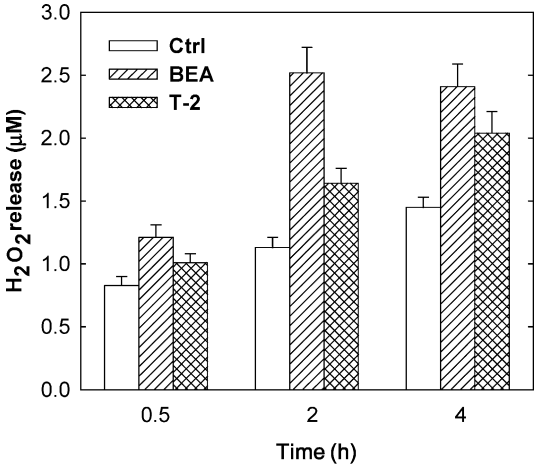

Fig. 2. H_2O_2 release in the incubation medium by tomato protoplasts treated with 50 µM BEA or T-2. At the indicated times, 1 ml of protoplasts suspension was taken for the determination of H_2O_2 concentration. Values represent the mean of four experiments ±SE.

(after 4 h by about 66 and 40% in BEA and T-2, respectively). Both mycotoxins induced a similar increase in H_2O_2 inside the cells, which was revealed by an increase in fluorescence of rhodamine of the treated cells (data not shown).

In addition to H_2O_2 determination, the oxidative damage in T-2 treated protoplasts, was monitored by measuring lipid peroxidation. As reported in Table 1, after 1 h of T-2 treatment a notable increase in lipid peroxidation occurred.

3.3. Determination of ascorbate and dehydroascorbate

While no significant difference in ASC and DHA contents occurred in control protoplasts during the time of the analysis, the treatment with 50 µM BEA resulted in a rapid and sharp decrease in the ASC content in the protoplasts (Fig. 3A). After 1 h of treatment the ASC content of BEA-treated protoplasts was 66% lower than that of the control and further decreases occurred with time (15 and 9% residual content after 2 and 3 h, respectively). Conversely, BEA did not induce a parallel increase in DHA content (Fig. 3B), the difference of which from that of the control remained insignificant throughout treatment. Consequently, the total

Table 1
Effect of 50 µM T-2 mycotoxin on lipid peroxidation

Time (h)	Lipid peroxidation (nmol MDA) $(1\times10^6$ protoplasts$)^{-1}$	
	Control	T-2
0.5	38.3±3.1	42.6±5.0
1	43.2±4.2	83.5±7.1**
3	50.4±6.1	157.1±11.3**
6	62.4±6.3	142.3±13.1**

Values represent the mean of four experiments ±SE. ** indicates values significantly different from the control by Student's *t* test with $P<0.01$.

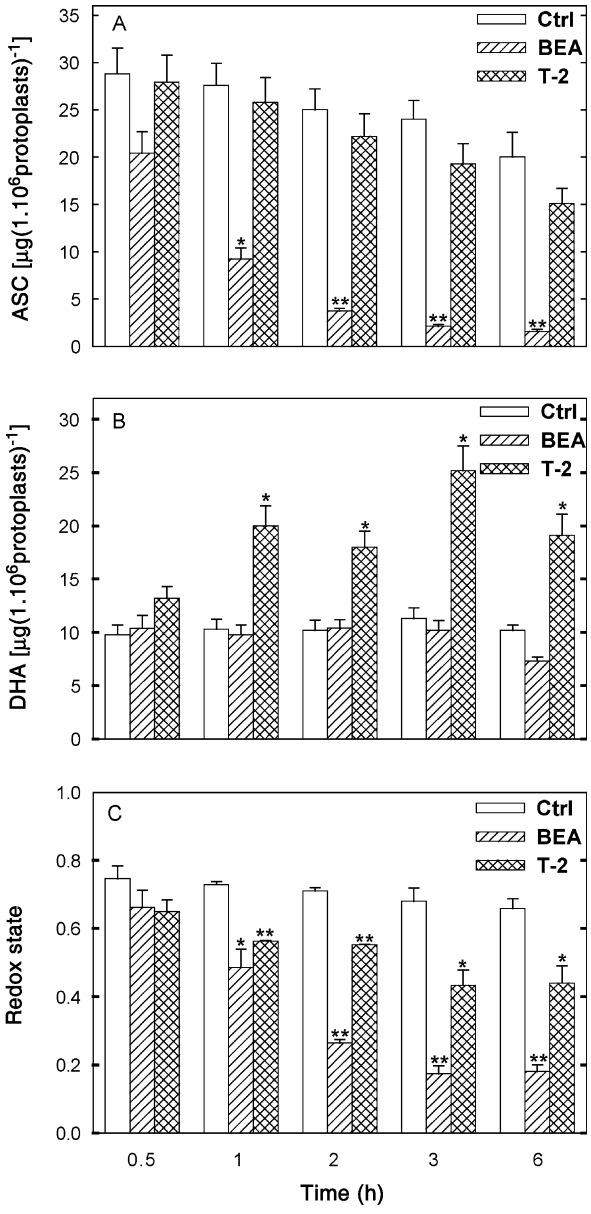

Fig. 3. Changes in ASC (A), DHA (B) content and in the redox state of the ascorbate pool (ascorbate/ascorbate + dehydroascorbate) (C) in tomato protoplasts treated with 50 µM BEA or T-2. Both treatments induced a decrease in ascorbate redox ratio. Values represent the mean of five experiments ±SE. * and ** indicate values significantly different from the control by Student's *t* test with $P<0.05$ and 0.01, respectively.

ascorbate pool (ascorbate plus dehydroascorbate), and its redox state (ascorbate/ascorbate + dehydroascorbate) were significantly lower than those of the control protoplasts (with $P<0.05$ at 1 h and with $P<0.01$ thereafter; Fig. 3C).

Differently from the treatment with BEA, the treatment with T-2 did not result in a significant change in the ASC level (Fig. 3A). However, T-2 induced a strong and progressive increase in DHA production (Fig. 3B). After such treatment the DHA contents were 94 and 123% higher than the control after 1 and 3 h, respectively. Therefore, the total ascorbate pool increased even if the ratio of reduced to

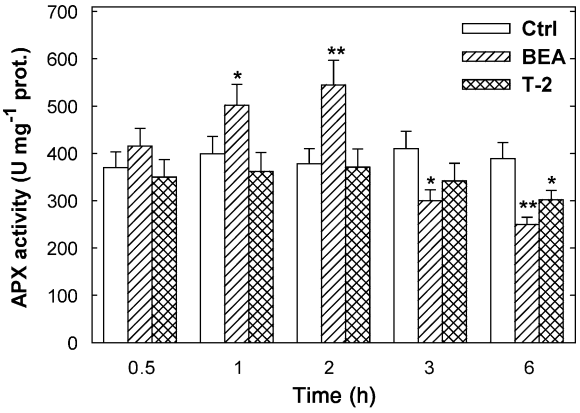

Fig. 4. APX activity in tomato protoplasts treated with 50 µM BEA or T-2. Values represent the mean of five experiments ±SE. 1 U = 1 nmol ASC oxidized min^{-1}. * and ** indicate values significantly different from the control by Student's *t* test with $P<0.05$ and 0.01, respectively.

oxidized ascorbate decreased with respect to the control (with $P<0.01$ at 1 and 2 h and with $P<0.05$ thereafter; Fig. 3C).

3.4. APX, DHAR and GLDH activities

The activities of APX and DHAR were assayed in order to verify whether the toxins also affected the enzymes of the ascorbate system of the protoplast.

Fig. 4 shows that APX activity transiently increased during the first 2 h of treatment with 50 µM BEA. On the contrary, no significant change in APX activity was found following treatment with 50 µM T-2 (Fig. 4). A parallel increase in DHAR activity was also evident in the first hours of treatment with BEA (Fig. 5). On the other hand, treatment with T-2 did not significantly change DHAR activity (Fig. 5).

To verify whether 50 µM BEA and T-2 toxins affected ASC biosynthesis in protoplasts, the activity of GLDH,

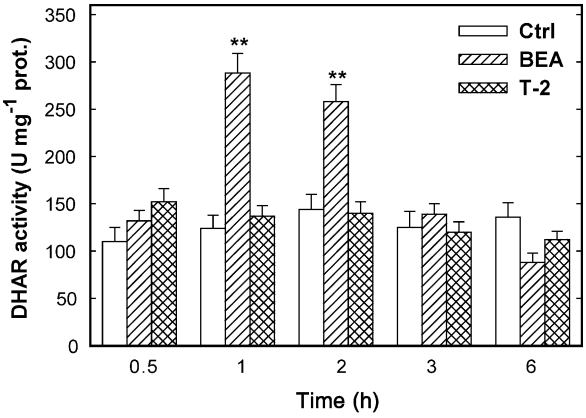

Fig. 5. DHAR activity in tomato protoplasts treated with 50 µM BEA or T-2. Values represent the mean of five experiments ±SE. 1 U = 1 nmol ASC formed min^{-1}. ** indicates value significantly different from the control by Student's *t* test with $P<0.01$.

Table 2
GLDH activity in tomato protoplasts treated with 50 μM BEA or T-2

Time (h)	Galactono-1,4-lactone dehydrogenase (nmol cytochrome *c* reduced min^{-1} mg^{-1} prot.)		
	Control	BEA	T-2
0.5	73±6	65±5	79±7
1	65±5	36±3**	90±9*
3	57±6	30±3**	85±9*
6	51±5	13±2**	75±7**

Values represent the mean of five experiments ±SE. * and ** indicate values significantly different from the control by Student's *t* test with $P<0.05$ and 0.01, respectively.

the enzyme which catalyzes the final step of ASC biosynthesis, was analyzed. Table 2 shows that the GLDH activity in T-2 treated protoplasts was significantly higher than that of the control protoplasts after 1 h, and remained constant thereafter. On the contrary, the treatment with BEA induced a progressive decrease in GLDH activity (Table 2).

3.5. Other antioxidants

To evaluate whether alternative antioxidant compounds were affected by toxin treatments, the levels of reduced and oxidized glutathione as well as catalase activity were analyzed. No significant difference in GSH and GSSG contents (data not shown) were found either with BEA or with T-2 treatment with respect to the control. No significant change in catalase activity was observed in protoplasts treated with T-2, while BEA treatment resulted in a sharp decrease in catalase activity (Table 3).

4. Discussion

The data reported in this study indicate that both toxins induce premature protoplast death but the phytotoxic action of BEA is higher than that of T-2, as shown by the more remarkable and rapid reduction of protoplast viability occurring under BEA-treatment (Fig. 1A and B). The cell death caused by 50 μM BEA on tomato protoplasts was almost complete within 6 h of treatment, while that of T-2 at the same time and concentration was much lower. The phytotoxicity of BEA has been reported to be effective at micromolar concentrations in other plant and animal systems [8,10]. It is known that BEA acts on biological membranes as an efficient ionophor for monovalent and divalent cations [35,36]. Indeed, in the presence of this mycotoxin, cells undergo an upsetting of the cellular cations (e.g. K^+ and Na^+, Ca^{++}) some of which play important roles in metabolism regulation [37].

Table 3
Catalase activity in tomato protoplasts treated with 50 μM BEA or T-2

Time (h)	Catalase (nmol H_2O_2 dismutated min^{-1} mg^{-1} prot.)		
	Control	BEA	T-2
0.5	95±8	84±7	89±8
1	89±9	43±3**	79±7
3	80±7	10±1**	74±6
6	51±5	8±1**	61±6

Values represent the mean of five experiments ±SE. ** indicates values significantly different from the control by Student's *t* test with $P<0.01$.

Even if many phytotoxins induce ROS generation, which in turn causes an oxidative stress in the phytotoxin treated cells [17], there is no clear evidence suggesting that BEA and T-2 activate ROS generating systems. We show that BEA and T-2 toxin produce increased levels of H_2O_2 (Fig. 2). Thus, our results suggest the involvement of an oxidative stress in the damage caused by T-2 and BEA. Moreover, the increase in DHA and in lipid peroxidation observed under T-2 treatments clearly indicates that the treated protoplasts undergo oxidative damage (Fig. 3B and Table 1). The defence response activated in tomato protoplasts by the presence of T-2, should be an increase in ASC biosynthesis, since a rise in the total pool of vitamin C (ASC+DHA) and an increase in GLDH activity occurs (Fig. 3A and B and Table 2), whereas the activity of APX and DHAR are not affected at all (Figs. 4 and 5). The increase in DHA content within cells could have negative metabolic effects contributing to the damage induced by this mycotoxin. Indeed it has been demonstrated that a rise in the DHA content induces the oxidation of several thiol-containing proteins and hence inhibits many enzymes [38]. Interestingly, one of the effects of T-2 is to bind proteic –SH groups, a process that inactivates certain thiol-containing enzymes [39,40]. Thus the increase in DHA generation could strengthen the T-2 damaging effect on –SH containing proteins.

In the case of BEA toxicity, the presence of oxidative stress is also supported by an increase in APX activity that is, however, only transient (Fig. 4). The more severe damage induced by this toxin on tomato protoplasts is also reflected by its effect on the ASC system. The strong depletion in ASC does not seem to be due to a net increase in its oxidation, since no rise in DHA was observed (Fig. 3B). The higher ASC oxidation due to the increase in APX activity is probably compensated by the parallel increase in DHAR. Due to the fact that BEA affects cell permeability, we can speculate that a certain amount of ASC is lost from the protoplast treated with this phytotoxin. The release of ASC from the cytosol to the apoplast could have some interesting repercussions for the penetration of phytopathogens in plant tissues. Indeed, it has been reported that in the cell wall, the presence of ASC causes the production of OH, the most powerful radical generated in biological systems [41]. This reactive species causes the loosening of the plant cell wall by non-enzymatic scission of the matrix polysaccharides [42]. Therefore, the release of ASC from the cytosol to the apoplast induced by BEA could facilitate pathogen penetration by increasing cell wall plasticity. Alternatively, we can also speculate that the ionophoric

action of BEA [43] interferes with ASC biosynthesis by altering the mitochondrial membranes. In fact, GLDH, the last enzyme of ASC biosynthesis is located in the inner mitochondrial membrane and, moreover, its activity requires a functional mitochondrial electron flow [44].

As regards the GSH system, in contrast to ASC, the preliminary data of this study seem to indicate that the involvement of this system in the defence response of protoplasts versus phytotoxicity of BEA and T-2 mycotoxins is not important. However, further investigations are necessary to support this thesis.

As far as the ROS-scavenging enzyme catalase is concerned, the decrease in activity induced by BEA treatment contributes to the maintaining of high H_2O_2 concentrations and to protoplast death. In this respect T-2 is less effective since it does not induce any change in catalase activity compared to control.

In conclusion, our data suggest that both BEA and T-2 cause protoplast damage, inducing their death, although differently affecting plant cell metabolism. In both cases the ascorbate system is altered, thus confirming the relevance of this metabolite in the perception and signal transduction of unfavourable environmental conditions [45,46]. Our results also confirm that the protoplast can be a useful tool for studying mycotoxin phytotoxicity and for ascertaining whether and how a single metabolite affects cellular metabolism in plants.

Acknowledgements

The authors thank Mrs L. Bianco (DPBP, University of Bari) for her valuable technical assistance.

References

[1] Marasas WFO, Nelson PE, Toussoun TA. Toxigenic *Fusarium* species: identity and mycotoxicology. Philadelphia, PA: Pennsylvania State University Press; 1984.
[2] Logrieco A, Moretti A, Castella G, Kosteck M, Golinski P, Ritieni A, et al. Beauvericin production by *Fusarium* species. Appl Environ Microbiol 1998;64:3084–8.
[3] Vey A, Quiot J-M, Vago C. Mise en évidence et étude de l'action dune mycotoxine, la beauvericine, dur des cellules d'insectes cultivées in vitro. Comp Rend Acad Sci Paris 1973;276:2489–92.
[4] Hamil RL, Higgens CE, Boaz HE, Gorman M. The structure of beauvericin, a new depsipeptide antibiotic toxic to *Artemia salina*. Tetrahedron Lett 1969;49:4255–8.
[5] Ojcius DM, Zychlinsky A, Zheng LM, Young D-EJ. Ionophore-induced apoptosis: role of DNA fragmentation and calcium fluxes. Exp Cell Res 1991;197:43–9.
[6] Tomoda H, Huang X-H, Cao J, Nishida H, Nagao R, Okuda S, et al. Inhibition of acyl-CoA: cholesterol acyltransferase activity by cyclodepsipeptide antibiotics. J Antibiot (Tokyo) 1992;45:1626–32.
[7] Gupta S, Kranoff SB, Underwood NL, Renwick JAA, Roberts DW. Isolation of beauveuricin as an insect toxin from *Fusarium semitectum* and *Fusarium moniliforme* var. subglutinans. Mycopathologia 1991; 115:185–9.
[8] Que FG, Gores GJ, La Russo NF. Development and initial application of an in vitro model of apoptosis in rodent cholangiocytes. Am J Physiol 1997;2772:G106–G15.
[9] Moretti A, Belisario A, Tafuri A, Ritieni A, Corazza L, Logrieco A. Production of beauvericin by different races of *Fusarium oxysporum* F. sp. *melonis*, the *Fusarium* wilt agent of muskmelon. Eur J Plant Pathol 2002;108:661–6.
[10] Sagakuchi M, Moretti A, Endo E, Matsuda Y, Toyoda H, Ouchi S. An approach to the use of plant sensitivity for simple detection of mycotoxin. Proceedings of First Asian Conference of Plant Pathology, Kuala Lumpur, Malaysia; August 2000. p. 262–79.
[11] Karppanen E, Rizzo A, Saari L, Berg S, Bostrom H. Investigation on trichothecene stimulated lipid peroxidation and toxic effects of trichothecenes in animals. Acta Veter Scand 1989;30:391–9.
[12] Rizzo AF, Atroshi F, Ahotupa M, Sankari S, Elovaara E. Protective effect of antioxidants against free-radical mediated lipid peroxidation induced by DON or T-2 toxin. J Vet Med Assoc 1994;41:81–90.
[13] Jelinek CF, Pohland AE, Wood G. Worldwide occurrence of mycotoxins in food and feeds—an update. J Assoc Off Anal Chem 1989;72:223–30.
[14] Marasas WFO, Smalley EB, Bamburg JR, Strong FM. Phytotoxicity of T-2 toxin produced by *Fusarium tricinctum*. Phytopathology 1971; 61:1488–91.
[15] Madhyastha MS, Marquardt RR, Abramson D. Structure–activity relationships and interactions among trichothecene mycotoxins as assessed by yeast bioassay. Toxicon 1994;32:1147–52.
[16] Mittler R. Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 2002;7:405–10.
[17] Heiser I, Oßwald W, Elstner EF. The formation of reactive oxygen species by fungal and bacterial phytotoxins. Plant Physiol Biochem 1998;36:703–13.
[18] De Gara L, de Pinto MC, Tommasi F. The antioxidant systems vis-à-vis reactive oxygen species during plant–pathogen interaction. Plant Physiol Biochem 2003;41:863–70.
[19] Noctor G, Foyer CH. Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol 1998; 49:249–79.
[20] Paciolla C, De Gara L, De Tullio MC, Arrigoni O. Distribution of cytosolic ascorbate peroxidase in angiosperms. Giornale Botanico Italiano 1996;130:729–37.
[21] Shigeoka S, Ishikawa T, Tamoi M, Miyagawa Y, Takeda T, Yabuta Y, et al. Regulation and function of ascorbate peroxidase isoenzymes. J Exp Bot 2002;53:1305–19.
[22] Karpinski S, Escobar C, Karpinska B, Creissen G, Mullineaux PM. Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in Arabidopsis during excess light stress. Plant Cell 1997;9:627–40.
[23] de Pinto MC, Tommasi F, De Gara L. Changes in the antioxidant systems as part of the signaling pathway responsible for the programmed cell death activated by nitric oxide and reactive oxygen species in tobacco Bright-Yellow 2 cells. Plant Physiol 2002;130: 698–708.
[24] Asada K. The water–water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons. Annu Rev Plant Physiol Plant Mol Biol 1999;50:601–39.
[25] March G, Tremolieres A. Protoplast growth and photoregulation. In: Pilet PE, editor. The physiological properties of plant protoplast. New York: Springer; 1985. p. 258–66.
[26] Kanai R, Edwards GE. Purification of enzymatically isolated mesophyll protoplasts from C3, C4 and Crassulacean acid metabolism plants using an aqueous dextran-polythylene glycol two-phase system. Plant Physiol 1973;52:484–90.
[27] Royall JA, Ischiropoulos H. Evaluation of 2′,7′-dichlorofluorescein and dihydrorhodamine 123 as fluorescent probes for intracellular H_2O_2 in cultured endothelial cells. Arch Biochem Biophys 1993;302: 348–55.

[28] Bellincampi D, Dipierro N, Salvi G, Cervone F, De Lorenzo G. Extracellular H_2O_2 induced by oligogalacturonides is not involved in the inhibition of the auxine-regulated rolB gene expression in tobacco leaf explants. Plant Physiol 2000;122:1379–85.
[29] Zhang J, Kirkham B. Antioxidant responses to drought in sunflower and sorghum seedlings. New Phytologist 1996;132:361–73.
[30] De Gara L, Paciolla C, De Tullio M, Motto M, Arrigoni O. Ascorbate-dependent hydrogen peroxide detoxification and ascorbate regeneration during germination of a high productive maize hybrid: evidence of an improved detoxification mechanism against reactive oxygen species. Physiol Plant 2000;109:7–13.
[31] Dipierro S, Borraccino G. Dehydroascorbate reductase from potato tubers. Phytochemistry 1991;30:427–9.
[32] Beaumont F, Jouve HM, Gagnon J, Gaillard J, Pelmont J. Purification and properties of a catalase from potato tubers (*Solanum tuberosum*). Plant Sci 1990;72:19–26.
[33] Ôba K, Seiko I, Nishikawa M, Mizuno H, Yamamoto T. Purification and properties of L-Galactono-γ-lactone dehydrogenase, a key enzyme for ascorbic acid biosynthesis, from sweet potato roots. J Biochem 1995;117:120–4.
[34] Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
[35] Dorschner E, Lardy H. Specificity of ion transport induced by beaveuricin. Antimicrob Agents Chemother 1968;8:11–14.
[36] Hamilton JA, Steinrauf LK, Braden B. Beauvericin and divalent cations: crystal structure of the barium complex. Biochem Biophys Res Commun 1975;64:151–6.
[37] Lemmens-Gruber R, Rachoy B, Steininger E, Kouri K, Saleh P, Krska R, et al. The effect of the *Fusarium* metabolite beauvericin on electromechanical and physiological properties in isolated smooth and heart muscle preparations of guinea pigs. Mycopathologia 2000;149:5–12.
[38] Paciolla C, De Tullio MC, Chiappetta A, Innocenti AM, Bitonti MB, Liso R, et al. Short- and long-term effects of dehydroascorbate in *Lupinus albus* and *Allium cepa* roots. Plant Cell Physiol 2001;42:857–63.
[39] Ueno Y, Matsumoto H. Inactivation of some thiol enzymes by trichothecene mycotoxins from *Fusarium* species. Chem Pharma Bull 1975;23:2439–42.
[40] Ueno Y. Trichothecenes as environmental toxicant. Toxicology 1984;5:1–15.
[41] Fry SC. Oxidative scission of plant cell wall polysaccharides by ascorbate-induced hydroxyl radicals. Biochem J 1998;332:507–15.
[42] Schopfer P. Hydroxyl radical-induced cell-wall loosening in vitro and in vivo: implications for the control of elongation growth. Plant J 2001;28:679–88.
[43] Kouri K, Lemmens M, Lemmens-Gruber R. Beauvericin-induced channels in ventricular myocytes and liposomes. Biochim Biophys Acta Biomembr 2003;1609:203–10.
[44] Millar H, Mittova V, Kiddle G, Heazlewood JL, Bartoli CG, Theodoulou FL, et al. Control of ascorbate synthesis by respiration and its implications for stress responses. Plant Physiol 2003;33:443–7.
[45] de Pinto MC, Tommasi F, De Gara L. Enzymes of the ascorbate biosynthesis and ascorbate–glutathione cycle in cultured cells of tobacco Bright Yellow 2. Plant Cell Physiol 2000;38:541–50.
[46] Foyer CH, Noctor G. Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiol Plant 2003;119:355–64.