A different trend of antioxidant defence responses makes tomato plants less susceptible to beauvericin than to T-2 mycotoxin phytotoxicity

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A B S T R A C T
The response to the phytotoxicity of beauvericin (BEA) and T-2, two mycotoxins produced by various species of the Fusarium genus, was studied in tomato seedlings. The components of the ascorbate-glutathione system, as well as other antioxidant systems such as superoxide dismutase, peroxidase and catalase enzymes were examined to evaluate their contribution to the defence strategies that the tomato plants employ to counteract attacks by the pathogens which produce these mycotoxins. This paper outlines that the simultaneous mobilization of different defence systems is necessary to overcome the phytotoxicity of BEA in tomato plants. Structural modifications of the cell wall, such as enhanced lignification, and metabolic rearrangements brought about by intracellular antioxidant systems to decrease the ionophore effect of BEA in tomato plants are suggested.

1. Introduction
Mycotoxins are naturally occurring secondary metabolites produced by the mycelial structures of either toxigenic or non-toxigenic filamentous fungi. Beauvericin (BEA) and T-2 toxin are produced mainly by pathogenic Fusarium species with a great economic impact for the agriculture sector [1]. They are found in cereal grains and animal feeds and have been associated with human and animal diseases [2].

Beauvericin is a cyclic hexadepsipeptide and a selective cation ionophore. Besides its high toxicity to insects [3], BEA is also cytotoxic to mammalian cell tissues, and has been reported to cause apoptosis in both murine and human cell lines [4]. Moreover, BEA shows toxic effects on the contractility of guinea pig smooth muscle, and is a very potent channel-forming molecule inducing pores in biological membranes [5]. Moreover, BEA has been proposed as a biological control agent against insect pests which affect important crop plants [6].

T-2 is a mycotoxin belonging to the trichotheccene family. Similar to other trichotheccenes, in eukaryotic cells T-2 is a potent inhibitor of protein [7] and nucleic acid synthesis [8]. Other toxic effects of T-2 include the alteration of membrane transport since it blocks the transport of amino acids, nucleotides and glucose as well as the activity of Ca–K channels [9]. Because of its high toxicity and being produced by Fusarium sporotrichioides, a fungal species contaminating cereals, T-2 is a toxin that can cause serious problems not only of an economic nature but also for the health of domestic livestock and humans [10].

While in the literature there are many reports in which the high toxicity of these two mycotoxins towards animals and humans has been demonstrated [5,10], little is known about their effects on plants, in particular about their role in plant–pathogen interactions and there is still a lot to learn about their potential toxicity in plants. Recently, beauvericin and T-2 have been demonstrated to induce cell death and alteration to the ascorbate metabolism in tomato protoplasts [11]. It is known that one early plant response to pathogen infection is an oxidative burst characterized by the production of huge amounts of reactive oxygen species (ROS) [12] such as hydrogen peroxide (H₂O₂). The increase in these ROS has been found in the leaves of plants in response to pathogen infection, wounding and osmotic stress [13–15] and is considered a second messenger for the expression of defence genes [16]. In different plant–pathogen interactions the microbial phytotoxins are known to induce enhanced ROS production. This production is
under the control of antioxidant defences, such as detoxifying enzymes and low-molecular-weight antioxidants. In this respect an important role is played by enzymes such as superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) and components of the ascorbate-glutathione cycle [17]. In this cycle ascorbate (ASC), which is considered one of the major soluble antioxidant compounds actively synthesized in plant cells, is mainly oxidized by the enzymes ascorbate peroxidase (APX) and ascorbate oxidase into monodehydroascorbate (MDHA). MDHA can be reduced to ascorbate by MDHA reductase (MDHAR) with the help of NAD(P)H or spontaneously to produce dehydroascorbate (DHA), the fully oxidized form. DHA can be reduced to ascorbate by reduced glutathione (GSH) in a reaction catalysed by DHA reductase (DHAR). The network of reactions of this cycle also includes reconversion of oxidized glutathione (GSSG) into GSH by glutathione reductase (GR) with the help of NAD(P)H [18]. This study was focused on some aspects of the defence mechanisms employed by tomato plants in response to BEA and T-2. In particular, the differences between the antioxidant defence responses of the plants were studied to investigate both the roles that the two mycotoxins play during the invasion of plants by fungal pathogens and the defensive potential of plants to counteract their attack.

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. This stock solution was further diluted with water to obtain the concentration utilized in the experiments.

2.2. Plant material

Tomato seeds (Lycopersicon esculentum L cv. Marmande) were germinated at 23 ± 1 °C and 55–60% relative humidity, under white fluorescent light with a 14/10 h photoperiod. Ten to twelve day old plants were cut at the collar level and the shoots incubated with 50 μM T-2 or BEA for different times. The same amount of methanol as that present in the treated samples was added to control plants. After incubation the shoots were washed with distilled water and analysed.

2.3. Histochemical staining

Two micrometer thick transverse sections of tomato plant stems were cut at 2 cm above the collar with an Ultracut Reichert–Jung ultramicrotome and stained with 1% safranin dissolved in 70% ethanol. The presence of xylem lignin appeared red in colour. The sections were observed and photographed under a Leitz ortholux microscope.

2.4. Determination of ascorbate and glutathione pool content

Five grams of shoots of control, T-2 or BEA treated samples were ground with two volumes of cold 5% (w/v) metaphosphoric acid in a porcelain mortar. The homogenate was centrifuged for 15 min at 20 000g and the supernatant was collected for analysis of ascorbate and glutathione pool content according to Zhang and Kirkham [19].

2.5. Enzyme analysis

Five grams of shoots of each sample (control, T-2 or BEA treated) were homogenized at 4 °C in 50 mM Tris–HCl, pH 7.8, containing 0.3 mM mannitol, 1 mM EDTA, and 0.05% (w/v) cysteine (buffer A) in a 1/3 ratio (w/v). The homogenate was centrifuged at 1000g for 5 min. The supernatant was re-centrifuged for 20 min at 25 000g. The resulting supernatant, assayed as a cytosolic fraction, was desalted by dialysis against 50 mM Tris–HCl, pH 7.8, and used for spectrophotometric analysis.

For POD extra-cellular analysis, the pellet resulting from the first centrifugation step was resuspended in buffer A plus 1% Triton X100 and centrifuged at 1000g for 5 min. The pellet was washed three times in buffer A, centrifuged as described above and then resuspended and incubated for 20 min in 1 M of NaCl and centrifuged for 20 min at 20 000g. The supernatant obtained (cell wall preparation) did not show glucose–6-phosphate dehydrogenase activity, as assayed according to Lohr and Waller [20].

Spectrophotometric assays of cytosolic ascorbate peroxidase (APX) (–ascorbate: hydrogen peroxide oxidoreductase, EC 1.11.1.11) activity were carried out following the H2O2-dependent oxidation of ASC at 265 nm in a reaction mixture containing 50 μg of total proteins, 50 μM ASC, 90 μM H2O2 and 50 mM phosphate buffer, pH 6.5. The non-enzymatic H2O2-dependent oxidation of ASC was subtracted (extinction coefficient 14 mM−1 cm−1).

Spectrophotometric assays of DHAR (glutathione: dehydroascorbate reductase, EC 1.8.5.1) activity were carried out following the increase in absorbance at 265 nm due to the GSH-dependent production of ASC. The reaction mixture contained 50 μg of total proteins, 1 mM DHA, 2 mM GSH and 100 mM phosphate buffer, pH 6.3. The rate of non-enzymatic DHA reduction was subtracted (extinction coefficient 14 mM−1 cm−1).

CAT (hydrogen peroxide: hydrogen peroxide oxidoreductase, EC 1.11.1.6) activity was evaluated according to Beaumont et al. [21] with minor modifications measuring the rate of H2O2 oxidation at 240 nm in a reaction mixture containing 50 μg of total proteins, 0.1 M phosphate buffer, pH 7.0, and 18 mM H2O2 (extinction coefficient 23.5 mM−1 cm−1). The control was carried out in the absence of H2O2.

The activity of MDHAR (EC 1.6.5.4) was evaluated by measuring the oxidation rate of NADH at 340 nm in a reaction mixture containing 50 μg of total proteins, 50 mM Tris–HCl buffer, pH 8, 0.2 mM NADH, 1 mM ASC and one unit of ASC oxidase (Sigma–Aldrich Chemie, Steinheim, Germany) (extinction coefficient 6 mM−1 cm−1). The rate of non-enzymatic reaction was taken into account.

Glutathione reductase (NADPH: glutathione disulphide oxidoreductase, EC 1.6.4.2) activity was measured as reported by Foster and Hess [22]. The rate of non-enzymatic GSSG reduction was subtracted.

Total peroxidase activity (POD) (EC 1.11.1.7) was measured according to Ferrer et al. [23] using 4–methoxy–α-naphthol (MN) as a substrate. The rate of non-enzymatic reaction was taken into account.

Enzymatic oxidation of coniferyl alcohol by POD was measured according to Pomar et al. [24]. The control was carried out in the absence of H2O2.

SOD (EC 1.15.1.1) activity was assayed according to McCord and Fridovich [25]. The protein content was determined according to Bradford [26], using bovine serum albumin as a standard.

2.6. Lipid peroxidation

Plant material was ground with four volumes of 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 10 000g for 10 min. One milliliter of the supernatant was diluted with 1 mL of 20% trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid. The level of lipid peroxidation was measured in terms of the malondialdehyde content determined by the thiobarbituric acid reaction described by Zhang and Kirkham [19].
2.7. Measurement of hydrogen peroxide

The H$_2$O$_2$ level in the extra-cellular medium was measured according to Bellicampi et al. [27]. The intracellular H$_2$O$_2$ concentration was evaluated according to Lee and Lee [28].

2.8. Statistical analyses

Statistical analyses of the differences between the mean values measured for the control and treated plants were performed using the Student’s t test. Differences were considered to be significant at $P < 0.05$ and highly significant at $P < 0.01$.

3. Results

3.1. Effects on the survival of the plants

In all our experiments a 50 $\mu$M concentration of the toxins was used because a similar concentration is produced by various Fusarium species in various plant matrices [4,29] and has been utilized in other systems [30]. Moreover, at a higher concentration (100 $\mu$M) the effects of BEA and T-2 were very strong and any effect was very difficult to study.

The two toxins induced different effects on the survival of the plants. After 3 h of treatment with 50 $\mu$M T-2, plants showed symptoms of wilting and at 24 h the plants were completely wilted compared to the control. The trend observed with the plants incubated with BEA was very different: symptoms of wilting were not shown even after 24 h of treatment (data not shown).

3.2. Xylem modifications

In order to verify whether the wilting was due to structural alterations, transverse sections of the stem were cut, stained and analysed by light microscopy to evaluate xylem cell wall lignification (Fig. 1). T-2 treated stems (Fig. 1C) showed scarcely lignified vessel walls as compared with the control (Fig. 1A). On the other hand in the BEA treated plant xylem (Fig. 1B), vessel cell walls were more lignified than in the control (Fig. 1A).

3.3. Analyses of the non-enzymatic components of the ASC-GSH cycle

A significant increase in both the ascorbate and dehydroascorbate content following the treatment with T-2 was found starting from 12 h (Fig. 2A and B) with a decrease in the ascorbate redox ratio (ASC/ASC plus DHA) (Fig. 2C). No changes were found in the content of the two metabolites in plants treated with BEA (Fig. 2A and B) nor was the ascorbate redox ratio changed (Fig. 2C).

As regards the glutathione content, a significant increase in reduced glutathione was found after 12 h in plants treated with BEA (Fig. 3A) while no change in the oxidized glutathione occurred (Fig. 3B). This did not lead to any change in the redox state that appeared similar to the control (Fig. 3C). On the other hand, the redox state sharply decreased after 12 h of treatment with T-2 (Fig. 3C) because of both a significant increase in the level of oxidized glutathione (Fig. 3B) and a decrease in reduced glutathione (Fig. 3A).

3.4. Analyses of enzymatic components of the ASC-GSH cycle

The activities of the cytosolic redox enzymes of the ASC-GSH cycle are reported in Fig. 4. The treatment with BEA induced a gradual increase in DHAR activity that became significant after 12 h as compared with the control. Differently from BEA, T-2 treatment induced a progressive decrease in DHAR activity that was significant at 24 h (Fig. 4A). BEA determined a significant increase in ascorbate peroxidase activity at every time of the analysis, while plants treated with T-2 exhibited a transient increase in APX activity with respect to the control at 6 and 12 h followed by a reduction at 24 h (Fig. 4B). No significant difference occurred in the activity of MDHAR in BEA treated plants. On the contrary, T-2 caused a significant decrease with respect to the control beginning after 12 h (Fig. 4C).

No significant difference in GR activity was induced by either mycotoxin (data not shown).

3.5. Analyses of other antioxidant systems

Table 1 shows a progressive increase in catalase activity in plants treated with BEA. This increase reached the maximum level at 24 h. On the other hand, a decrease in activity was observed in T-2 treated plants after 24 h.
Total peroxidase activity in the cytosolic fraction, measured using the non-physiological substrate 4-methoxy-α-naphthol, was different in the treated samples, as compared with the control. As shown in Fig. 5A, BEA induced a significant increase in POD activity as compared with the control beginning from 6 h while T-2 induced a slight decrease in activity throughout the treatment. Cell wall-associated (extra-cellular) peroxidases showed a similar trend to cytoplasmic peroxidases (data not shown). Moreover, the activity of cell wall POD capable of oxidizing coniferyl alcohol was measured. As shown in Fig. 5B the specific peroxidase activity involved in lignification increased after BEA treatment while it showed a remarkable decrease after T-2 treatment, throughout the analysed period.

As regards the SOD analysis, no significant difference in the enzymatic activity in BEA and T-2 treated plants with respect to the control occurred (Fig. 6).

### 3.6. H2O2 production

As shown in Table 2, both the intracellular and the extra-cellular concentrations of H2O2 in treated plants were higher than in the control. In particular, T-2 induced a significantly higher increment at every time of the analysis with respect to BEA which instead determined a significant increase only at 24 h.

### 3.7. Lipid peroxidation

Since it is known that some mycotoxins induce alterations in the membrane structure, the effect of T-2 and BEA on lipid peroxidation was also examined. T-2 induced an increase in lipid peroxidation, measured in terms of malondialdehyde concentration, after 12 h (Table 3), while no significant difference occurred in the BEA treated plants with respect to the control (data not shown).

### 4. Discussion

The roles of the ASC-GSH cycle and other antioxidants, such as POD, SOD and CAT, have been examined in tomato plants treated with BEA and T-2 mycotoxins to improve understanding of the action mechanisms of these phytotoxins produced by Fusarium species and responsible for the development of important diseases.
The reported data indicate that both T-2 and BEA mycotoxins are phytotoxic for tomato plants. However T-2 is more phytotoxic than BEA, as demonstrated by the partial and complete wilting of the T-2 plants after 3 and 24 h respectively. This wilting could also be due to some modifications of the anatomical features of the xylem. In fact, histochemical analysis revealed the almost complete absence of lignin in the cell walls of the xylem tissue in T-2 treated plants (Fig. 1C) with respect to the control (Fig. 1A). Lignin is a phenolic polymer that plays an important role in water transport and in reinforcing the walls of the vascular cells of higher plants during plant–pathogen interaction [31]. So, it can be speculated that in plant–pathogen interactions in which the phytopathogens are T-2 producing species of the *Fusarium* genus, one of

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### Table 1

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Catalase specific activity</th>
<th>BEA</th>
<th>T-2</th>
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<td></td>
<td>nmoles of H$_2$O$_2$ oxidized min$^{-1}$ mg$^{-1}$ prot</td>
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<tr>
<td>Control</td>
<td>106 ± 8</td>
<td>111 ± 10</td>
<td>99 ± 10</td>
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<td>6</td>
<td>103 ± 9</td>
<td>126 ± 9</td>
<td>96 ± 8</td>
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<td>12</td>
<td>100 ± 9</td>
<td>128 ± 6*</td>
<td>86 ± 9</td>
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<tr>
<td>24</td>
<td>95 ± 7</td>
<td>140 ± 8*</td>
<td>72 ± 6*</td>
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</table>

Values represent the mean of at least four experiments ± SE.

* Values significantly different from the control according to the Student’s t test with *P* < 0.05.

** Values significantly different from the control according to the Student’s t test with **P** < 0.01.

### Fig. 4.

Activities of the redox enzymes DHAR (A), APX (B) and MDHAR (C) in tomato control (Ctrl) plants and those treated with 50 μM BEA or T-2. Values represent the mean of at least four experiments ± SE. * and ** indicate values significantly different from the control according to the Student’s t test with *P* < 0.05 and **P** < 0.01, respectively. 1 U = 1 nmol of the substrate metabolized min$^{-1}$.

### Fig. 5.

POD activity in the cytoplasm (A) and in the cell walls (B) of tomato control (Ctrl) plants and those treated with 50 μM BEA or T-2. Values represent the mean of at least four experiments ± SE. * and ** indicate values significantly different from the control according to the Student’s t test with *P* < 0.05 and **P** < 0.01, respectively. For cytoplasmic POD (A), 1 U = 1 nmol of MNB oxidized min$^{-1}$; for cell wall POD (B), 1 U = 1 nmol of coniferyl alcohol oxidized min$^{-1}$.

### Fig. 6.

SOD activity in tomato control (Ctrl) plants and those treated with 50 μM BEA or T-2. Values represent the mean of at least four experiments ± SE. 1 U = the amount of enzyme required to inhibit the reduction rate of NBT by 50% at 25°C.
The roles of this mycotoxin could be to bind to lignin and so to destroy it, thus facilitating the passage of the pathogen into the host cell. This hypothesis is supported by reports [32] indicating that, when T-2 and lignin rich foodstuffs were both added to their diet, rats overcame feed refusal and growth depression due to T-2 toxin, which was explained by a bond between the T-2 toxin and lignin in the intestinal lumen that blocks absorption of the toxin. Differently from the T-2 treated plants, after BEA treatment the vessels appeared more lignified than in the control plants (Fig. 1). The increase in vessel lignification could be explained by the increased peroxidase activity observed in plants treated with BEA (Fig. 5B). In fact, it is known that POD is involved in the oxidative polymerization of lignin precursors and its role in the lignification of xylem vessels is widely accepted [33]. Moreover, the increase in POD activity (Fig. 5) could also be involved in the cross-linking of cell wall components, inducing cell wall strengthening and stiffening. The involvement of POD in cell wall strengthening has also been observed in various plant–pathogen interactions which provide a mechanical barrier against invasion by pathogens [34]. Therefore, enhanced lignification could be a precondition for the survival of plants: in T-2 treated plants the sharp decrease in the lignin content in the vessel walls (Fig. 1C) led to their collapse and consequently reduced water transport, loss of cell turgidity and wilting of the plant.

It is also known that POD is an important component in regulating the presence of H2O2 in the plant responses to stress where a concomitant increase in POD activity, H2O2 concentration and enhanced tolerance to the phytotoxic action performed by the toxin has been observed [35]. In our conditions this trend occurs in BEA treated plants; however in T-2 treated plants a higher level of H2O2 was also found but a decrease in POD activity occurred.

All our evidence indicates that the damage caused by T-2 is due to oxidative stress since T-2 induces increases in both the intracellular and extra-cellular H2O2 content (Table 2) and lipid peroxidation (Table 3). The overall defence response of the plants to the treatment with T-2 consists of an increase in the ASC content after 12 h (Fig. 2A) and the transient increase in ascorbate peroxidase after 6 h (Fig. 4B). However, this antioxidant defence response was not sufficient to counteract T-2 toxicity. In addition, the strong decrease in the activity of the enzymes that restore ascorbate to the reduced form (Fig. 4A and C), namely monodehydroascorbate reductase and dehydroascorbate reductase, with unchanged activity of glutathione reductase, contribute to maintaining high levels of DHA and GSSG, which are responsible for the cellular redox imbalance. In this respect, in tomato plants treated with T-2 we observed that severe wilting was preceded by a shift of the ASC and GSH redox pairs towards the oxidized forms (Figs. 2C and 3C), a cellular condition that has been reported to trigger programmed cell death [36]. BEA is reported to be phytotoxic for animal and plant systems and to induce oxidative stress at micromolar concentrations [3–5,11]. When treated with BEA our plants also did not show any symptom of suffering in the long term, thus indicating that they were able to counteract the phytotoxicity of the toxin.

An increase in H2O2 content was also found in the BEA treated plants (Table 2), so these plants also underwent oxidative stress. However, the significant increase in the enzymatic activities of APX and CAT after BEA treatment (Fig. 4B and Table 1) indicates that an adequate and efficient antioxidant defence response was activated in the plants. In this respect it should be noted that we observed a gradual increase in the content of GSH (Fig. 3A). This increase allows DHAR to carry out the reconversion of DHA to ASC more efficiently, since it utilizes GSH as an electron donor. The higher level in ASC, as a consequence of the rise in DHAR activity, compensates for the increased oxidation of ASC due to the increase in APX activity. This trend allows the balance of the redox state to be maintained in the BEA treated plants.

Therefore the survival of the plants treated with BEA seems to be due to additive effects at different cellular levels, that is to structural modifications of the cell wall (more lignification) as well as to metabolic rearrangements induced by intracellular antioxidant systems.

We also made an attempt to assess the possible differences between beauvericin-induced cytotoxicities in tomato protoplasts and in whole plant cells. It is known that some fungal toxins are cytolytic since they induce the formation of large pores in the host cell membranes so that the cells undergo uptake of important cations (e.g. Ca2+ and Na+) [37,38]. BEA has also been reported to be an efficient ionophore [39] and the structural similarity between BEA and enniatins led Wang et al. [40] to hypothesize that the action mechanism of this mycotoxin might involve the disruption of either the metabolism or some functions of sphingolipids. In a previous paper we reported that BEA, at micromolar concentrations, is phytotoxic for tomato protoplasts and is able to open pores in biological membranes [5,11]. In whole plants BEA seems unable to perform its ionophoric action. The cell wall could represent a valid tool for the plant cell to limit toxin diffusion. Consistently the increase in POD activity involved in lignification (Fig. 5B), as well as the increased lignin staining observed in plants treated with BEA (Fig. 1B), could be an early response to reinforce this constitutive defence barrier against phytotoxicity.

| Table 2 |

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<thead>
<tr>
<th>Intracellular (A) and extra-cellular (B) content of H2O2 in plants treated with 50 μM BEA or T-2 at different times</th>
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Values represent the mean of at least four experiments ± SE.

a Values significantly different from the control according to the Student’s t test with P < 0.05.

b Values significantly different from the control according to the Student’s t test with P < 0.01.

| Table 3 |

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<th>Effect of 50 μM T-2 mycotoxin on lipid peroxidation</th>
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<td>Time (h)</td>
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References

