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Phytotoxicity of the tetramic acid metabolite trichosetin

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Abstract

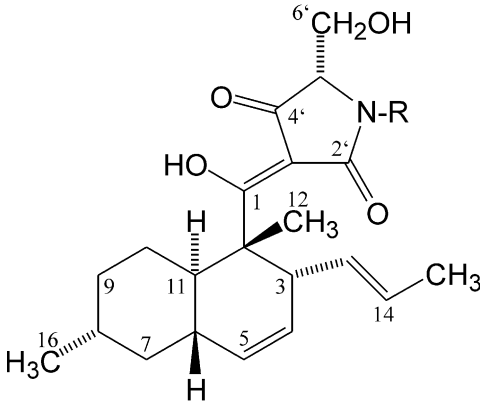
Trichosetin, a tetramic acid-containing metabolite produced in the dual culture of *Trichoderma harzianum* and *Catharanthus roseus* (L.) G. Don callus, was subjected to phytotoxicity assays. In seedling growth assays, trichosetin inhibited root and shoot growth of all five plant species tested by damaging the cell membrane, as evidenced by the dose-dependent increase in electrolyte leakage and lipid peroxidation. Vital staining of trichosetin-treated *Nicotiana tabacum* BY-2 cells, with rhodamine 123, showed a weaker green fluorescence compared to controls indicating damaging effects on mitochondria. FDA-PI staining, to determine cell viability, indicated that cells of the trichosetin-treated roots were mostly dead.

Keywords: Dual culture; Phytotoxicity; Tetramic acid; Trichosetin

1. Introduction

We recently reported the isolation of trichosetin **1**, the *N*-desmethyl homolog of equisetin **2** (Fig. 1), a *Fusarium* toxin (Burmeister et al., 1974), from the dual culture of *Trichoderma harzianum* and *Catharanthus roseus* callus (Marfori et al., 2002). Both equisetin (**2**) and trichosetin (**1**) exhibit remarkable antimicrobial activity against Gram-positive bacteria, such as *Staphylococcus aureus* and *Bacillus subtilis*. They belong to an important group of secondary metabolites having, in common, a tetramic acid moiety acylated at the 3-position. These naturally occurring tetramic acids have attracted a great deal of interest because of the very diverse biological functions exhibited by most of them (Royles, 1995).

While tetramic acid metabolites produced by various fungi have diverse biological activities, only two have been reported to be phytotoxic. Recently, Wheeler et al. (1999) found that equisetin (**2**) was phytotoxic, establishing it as a pathogenic factor of *Fusarium* spp. on seeds and seedlings. Apart from equisetin (**2**), the other is tenuazonic acid first isolated from *Alternaria tenuis* (Rosett et al., 1957). Since trichosetin (**1**) is very similar to equisetin (**2**) in structure and antimicrobial activity, we tested whether trichosetin (**1**) might also be phytotoxic. We now report the first details of the phytotoxicity of trichosetin (**1**).



(**1**): R= H

(**2**): R= CH_3

Fig. 1. Chemical structure of trichosetin (**1**) and equisetin (**2**).

2. Results and discussion

The effects of trichosetin (**1**) on seedling growth of various plants, as expressed by root and shoot length,

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are shown in Tables 1 and 2. Increased trichosetin (**1**) concentration decreased seedling growth. The maximum dose used (10.0 μg ml^{-1}), caused significant inhibition of both roots and shoots. In most plants, root growth was more inhibited than shoot growth. The effect of trichosetin on the reduction of seedling growth compared to tenuazonic acid was plant species- and organ-dependent. In *Oryza sativa*, *Medicago sativa* and *Capsicum frutescens*, trichosetin (**1**) was more effective than tenuazonic acid in reducing shoot growth, but less

Table 1
Effect of trichosetin (**1**) and tenuazonic acid on root length of various plants

Plant species	Concentration (μg ml^{-1})	Root length (mm)	Relative growth (% of control)
Oryza sativa			
Methanol control	–	68±3	100
Trichosetin	1.25	58±3	85
	2.50	42±3	62
	5.00	20±2	29
	10.00	9±1	13
Tenuazonic acid	1.25	25±2	37
	2.50	12±1	18
	5.00	4±1	6
	10.00	3±1	4
Vigna radiata			
Methanol control	–	46±4	100
Trichosetin	1.25	22±2	48
	2.50	20±2	44
	5.00	18±1	39
	10.00	15±1	33
Tenuazonic acid	1.25	40±2	87
	2.50	29±2	63
	5.00	29±4	63
	10.00	20±2	44
Medicago sativa			
Methanol control	–	34±2	100
Trichosetin	1.25	32±2	94
	2.50	22±2	65
	5.00	10±2	29
	10.00	6±1	18
Tenuazonic acid	1.25	30±2	88
	2.50	21±2	62
	5.00	11±2	32
	10.00	5±1	15
Capsicum frutescens			
Methanol control	–	30±2	100
Trichosetin	1.25	22±2	73
	2.50	19±2	63
	5.00	6±1	20
	10.00	2±1	7
Tenuazonic acid	1.25	8±1	27
	2.50	6±1	20
	5.00	5±1	17
	10.00	2±1	7
Lycopersicon esculentum			
Methanol control	–	44±2	100
Trichosetin	1.25	24±2	54
	2.50	8±1	18
	5.00	6±1	14
	10.00	4±1	9
Tenuazonic acid	1.25	40±2	91
	2.50	29±1	66
	5.0	27±1	61
	10.00	7±1	16

Table 2
Effect of trichosetin (**1**) and tenuazonic acid on shoot length of various plants

Plant species	Concentration (μg ml^{-1})	Shoot length (mm)	Relative growth (% of control)
Oryza sativa			
Methanol control	–	54±3	100
Trichosetin	1.25	47±3	87
	2.50	44±2	82
	5.00	44±4	82
	10.00	37±2	68
Tenuazonic acid	1.25	54±2	100
	2.50	51±2	94
	5.00	49±2	91
	10.00	44±2	82
Vigna radiata			
Methanol control	–	25±1	100
Trichosetin	1.25	24±2	96
	2.50	22±2	88
	5.00	10±1	40
	10.00	8±1	32
Tenuazonic acid	1.25	25±1	100
	2.50	24±2	96
	5.00	22±2	88
	10.00	18±2	72
Medicago sativa			
Methanol control	–	21±2	100
Trichosetin	1.25	20±2	95
	2.50	12±1	57
	5.00	10±1	48
	10.00	4±1	19
Tenuazonic acid	1.25	20±1	95
	2.50	18±1	86
	5.00	15±2	71
	10.00	7±1	33
Capsicum frutescens			
Methanol control	–	12±1	100
Trichosetin	1.25	9±1	75
	2.50	7±1	58
	5.00	5±1	42
	10.00	4±1	33
Tenuazonic acid	1.25	10±1	83
	2.50	9±1	75
	5.00	8±1	67
	10.00	4±1	33
Lycopersicon esculentum			
Methanol control	–	26±1	100
Trichosetin	1.25	25±1	96
	2.50	18±1	69
	5.00	6±1	23
	10.00	3±1	12
Tenuazonic acid	1.25	24±2	92
	2.50	22±2	85
	5.00	22±2	85
	10.00	7±1	27

effective than the latter in reducing root growth. The converse was true with *Vigna radiata* and *Lycopersicon esculentum*.

Different seedlings treated with varying trichosetin (**1**) concentrations are shown in Fig. 2a–e. Formation of necrotic lesions was very obvious in coleoptiles of *O. sativa* and in cotyledons of *V. radiata*. Treated seedlings did not resume growth when placed in water without trichosetin (**1**), indicating that seedling damage was permanent. However, trichosetin (**1**) failed to inhibit seed germination even at maximum dose used (data not shown).

Since trichosetin (**1**) caused a pronounced inhibition of root growth, and necrosis in coleoptile of *O. sativa*, the root and coleoptile sections of *O. sativa* were subjected to electrolyte leakage assays to determine possible damage to cell membranes. Results are shown in Fig. 3 a–d. When root or coleoptile sections were treated with trichosetin (**1**) under illuminated conditions, a rapid dose-dependent increase in the electrical conductivity of the assay media was observed. The increase in electrolyte leakage caused by trichosetin (**1**) was greater than that caused by the same concentration of paraquat, a known light-dependent plasma membrane disruptor (Dodge and Harris, 1970). Increased electrolyte leakage was similarly observed in dark-incubated tissues. In contrast, paraquat-induced electrolyte leakage in darkness was insignificant even after 48 h. This suggests that the action of trichosetin (**1**) is different from the light-dependent membrane damage caused by paraquat. Electrolyte leakage due to tenuazonic acid was also insignificant under both light and dark conditions, suggesting a totally different mode of action.

Generally, membrane damage is the result of lipid peroxidation (Tappel, 1973). A reliable method to measure the degree of lipid peroxidation is the 2-thiobarbituric acid assay (Kosugui and Kikugawa, 1989) by which TBA-reactive metabolites, e.g. malondialdehyde (MDA), signify the formation of polyunsaturated fatty acid hydroperoxides (Heath and Packer, 1968). Consequently, the TBA-reactive metabolites in coleoptile of *O. sativa* were analyzed. The results are shown in Fig. 4.

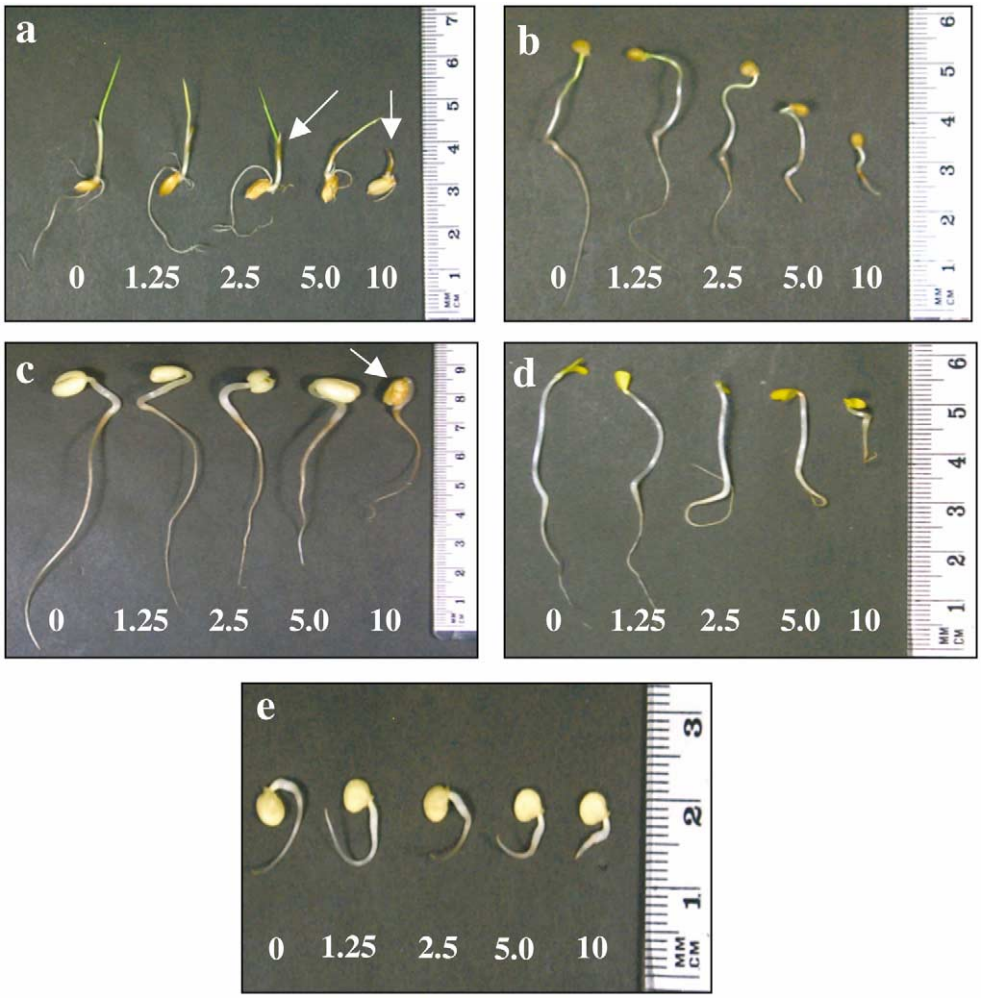

Fig. 2. Seedlings treated with 0, 1.25, 2.5, 5 and 10 g ml^{-1} trichosetin (**1**). Note the inhibition of root growth and formation of necrotic lesions indicated by the arrows. (a) *Oryza sativa*; (b) *Lycopersicon esculentum*; (c) *Vigna radiata*; (d) *Medicago sativa*; (e) *Capsicum frutescens*.

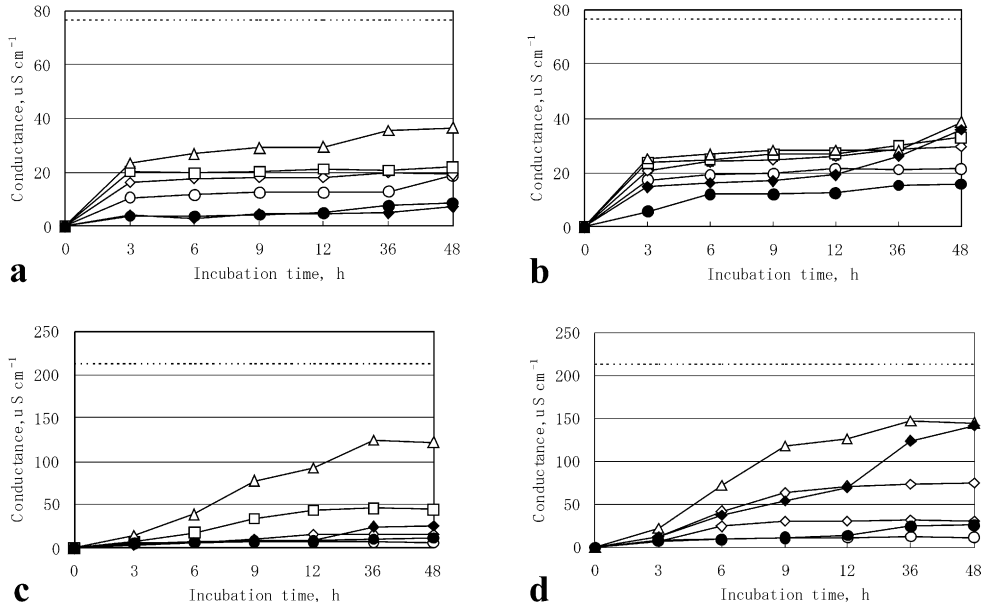

Fig. 3. Dose-response for electrolyte leakage in root and coleoptile sections of *Oryza sativa* after incubation in different concentrations of trichosetin (**1**) at various time intervals, as determined by change in conductivity of treatments minus conductivity of the Methanol control. Paraquat and tenuazonic acid were used as reference compounds. The dashed line represents maximum leakage obtained by boiling. (a) Root sections incubated under dark conditions, (b) root sections incubated under light conditions, (c) coleoptile sections incubated under dark conditions, and (d) coleoptile sections incubated under light conditions. Legends: 1.25 μg ml^{-1} trichosetin **1** (○); 2.5 μg ml^{-1} trichosetin **1** (◇); 5 μg ml^{-1} trichosetin **1** (□); 10 μg ml^{-1} trichosetin **1** (△); 10 μg ml^{-1} paraquat (◆); 10 μg ml^{-1} tenuazonic acid (●).

As compared with the control, treatment with trichosetin (**1**) increased the MDA content in coleoptile. This increase in MDA content in response to trichosetin (**1**) occurred in a dose-dependent manner suggesting that trichosetin (**1**) caused lipid peroxidation.

Lipid peroxidation may be due to excessive production of reactive oxygen species resulting from mitochondrial damage. This is very significant because equisetin (**2**), which is structurally-related to trichosetin (**1**), has been reported to affect rat liver mitochondria (Konig et al., 1993). To determine if trichosetin (**1**) damages plant mitochondria, *Nicotiana tabacum* L. BY-2 cells were treated with trichosetin (**1**), then stained with rhodamine 123, a specific vital stain for mitochondria (Wu, 1987). The results are shown in Fig. 5. Intense fluorescence in the control was due to the uptake of rhodamine 123 by active mitochondria. The trichosetin (**1**)-treated cells had a weaker green flourescence compared with the control suggesting that mitochondria of BY-2 cells were damaged by trichosetin (**1**). Experiments were also conducted to determine the effects of trichosetin (**1**) on the electron-transport chain, but the results showed that it has no effect on the various protein complexes of the electron-transport chain (data not shown). Thus, it is likely that trichosetin (**1**) is inhibiting the substrate anion carriers of the inner membrane, as has been observed with equisetin (**2**) (Konig et al., 1993).

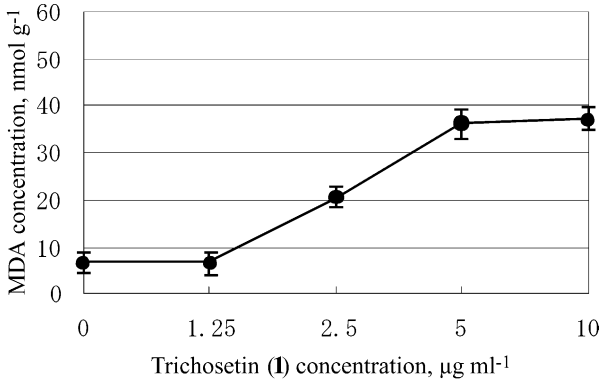

Fig. 4. Lipid peroxidation, expressed as MDA concentration, in coleoptile of *Oryza sativa* seedlings after 4 days of incubation in 0, 1.25, 2.5, 5 and 10 μg ml^{-1} trichosetin.

Finally, the effect of trichosetin (**1**) on cell viability was determined. *O. sativa* seedlings were grown hydroponically either in the presence of 10.0 g ml^{-1} trichosetin (**1**) or 62.5 l methanol as a control treatment. After 4 days, the roots were harvested and double-stained with fluorescein diacetate (FDA) and propidium iodide (PI). Viable cells showed green fluorescence due to FDA whereas dead cells were stained red with PI. Fig. 6a and b show *O. sativa* roots treated with and without trichosetin (**1**), respectively. Dead cells were observed in the root apical meristem, including the entire root cap of trichosetin (**1**) treated *O. sativa*. The epidermal cells in the upper region were also dead. These results suggest that trichosetin (**1**) can cause cell death.

From the above results, it can be concluded that trichosetin (**1**) is phytotoxic and its structural difference with equisetin (**2**), i.e. the methyl group attached to the

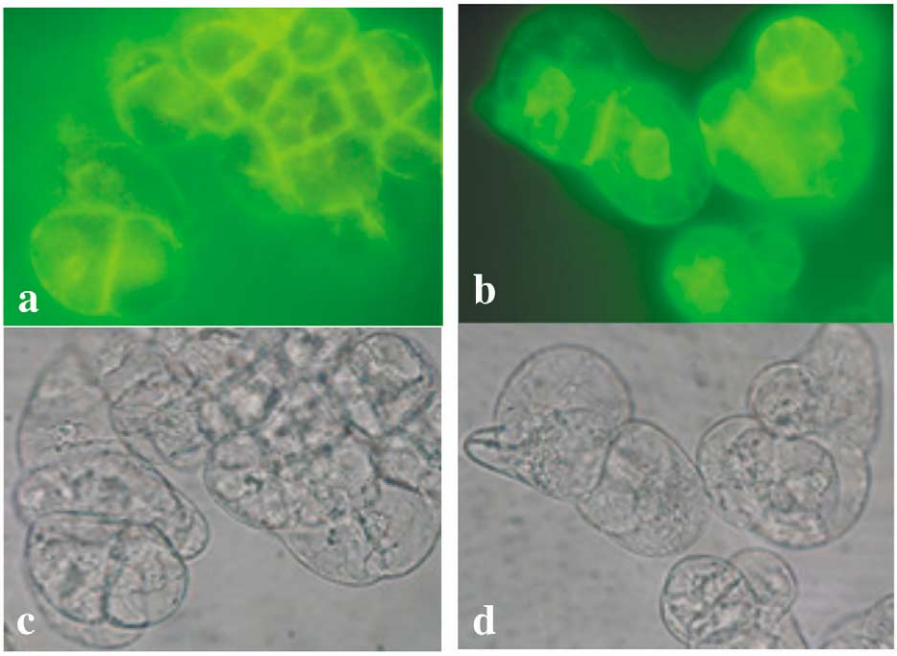

Fig. 5. Vital staining of *Nicotiana tabacum* BY-2 cells with rhodamine 123. Note the weaker green flourescence in trichosetin (**1**)-treated cells (a) as compared with that of the untreated control (b). Bright-field views of these cells are shown in (c) and (d), respectively.

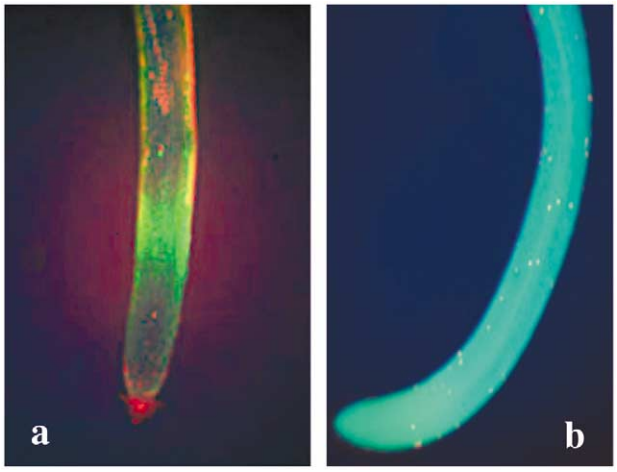

Fig. 6. Cell viability of *Oryza sativa* roots as determined by FDA–PI staining. Viable cells showed green fluorescence due to FDA while dead cells were stained red due to PI. (a) Trichosetin (**1**)- treated root tip, and (b) root tip of untreated control.

N atom, has little effect on phytotoxic activity. The mode of action of equisetin (**2**) is unknown, though Wheeler et al. (1999) suggested that it might have an effect on energy-related reactions in mitochondria based on previous studies showing the effect of equisetin (**2**) on energy-related reactions in chromatophores of *Rhodospirillum rubrum* (Nyren and Strid, 1989), mitochondria of *Saccharomyces cerevisiae* (Lundin et al., 1992) and rat liver mitochondria (Konig et al., 1993). Evidence presented in this study suggests a connection between plasma membrane and mitochondrial damage caused by trichosetin (**1**). Active mitochondria are predominantly present in meristematic tissues such as in root tips where the cell death occurred. Mitochondria have been well documented as the site where most radicals are produced in response to stress (Dionisi et al., 1975; Nohl and Hegner, 1978; Ambrosio et al., 1993; Dawson et al., 1993; Moller, 2001). It is also well known that these radicals are the primary agents of lipid peroxidation (Kappus, 1985; Bose et al., 1990). Membrane lipid peroxidation causes electrolyte leakage eventually leading to cell death.

In summary, the phytotoxicity of trichosetin (**1**) can be attributed to its damaging effect on mitochondria. A more detailed study dealing with the interaction of trichosetin (**1**) with submitochondrial components should be very interesting to understand better its mechanism of action.

3. Experimental

3.1. Isolation of trichosetin (*1*)

Isolation of trichosetin (**1**) from 7.5 kg dual culture of *C. roseus* callus and *T. harzianum* was effected (Marfori et al., 2002), with some modifications. Two weeks following fungal inoculation, infected calli were obtained and soaked in MeOH. The MeOH extract was evaporated and the residue (107.23 g) suspended in water. After adjusting the pH to 4.0, it was sequentially partitioned with *n*-hexane and EtOAc. The EtOAc extract (3.91 g) was applied to a silica gel column ($46.5 \times 6.5\ cm^2$) successively eluted with toluene (3000 ml)→ toluene:

Me_2CO (2700:300 → 2400:600 → 2100:900 → 1800:1200, ml/ml) → Me_2CO (3000 ml) → MeOH (6000 ml). The concentrated MeOH fraction (3.86 g) was subjected to MPLC (80 g ODS-A, 40 × 2.0 cm^2) eluted with MeCN:0.1%HCO_2H (A:B) in 200 ml each of 30, 40, 50, 60, 70, 80, 90 and 100% A:B. The 80% MeCN fraction (188 mg) was again subjected to another MPLC step (ODS-A 120-S150, 50 × 1 cm^2) using MeCN/0.1% HCO_2H (60:40) at a flow rate of 1.5 ml min^{-1}. Fractions were collected every 4 min. Fractions 41–60 were pooled (103.4 mg), and subjected to preparative HPLC (ODS-3, 10 × 250 mm I.D.) eluted isocratically with MeCN/0.1% HCO_2H (64:36) at 3 ml min^{-1} which afforded trichosetin **1** (68.7 mg) as the major peak with a retention time of 37 min.

3.2. Seedling growth assay

Seedlings of 5 plant species, *O. sativa*, *V. radiata*, *M. sativa*, *C. frutescens* and *L. esculentum*, were subjected to a seedling growth bioassay in liquid cultures following the method of Kuboi and Fujii (1984). Briefly, seeds were surfaced-sterilized and dark-germinated in distilled water in 250-ml Erlenmeyer flasks, with 80 rpm shaking. Germinated seedlings, with a combined root and shoot length of 3–5 mm, were selected and treated with trichosetin **1** at 1.25, 2.5, 5.0 and 10.0 μg ml^{-1}. All treatments consisted of 10 seedlings placed in a 125-ml Erlenmeyer flask with 25 ml distilled water containing trichosetin **1**, which was added to the water in 62.5 l MeOH. The treated cultures were then dark-incubated at 30 °C on a rotary shaker at 80 rpm, for 3–4 days. Tenuazonic acid (Sigma), purchased as a copper salt, was first passed through Dowex 50W-X8, H form (Dow Chemical Co.), and used as a positive control at 1.25, 2.5, 5.0 and 10.0 g ml^{-1}. Negative control was MeOH at 62.5 μl per 25 ml distilled water. Experiments were done in triplicate.

3.3. Electrolyte leakage assay

Electrolyte leakage assay utilised the method of Fukushima et al. (1998), with some modifications. Ten 8–10 mm sections of rice root, or coleoptile were soaked in 1 ml of 1% sucrose/1 mM MES–NaOH buffer (pH 6.5) containing varying concentrations of trichosetin **1** in the wells of a 24-well plate. Paraquat and tenuazonic acid, both at 10 g ml^{-1}, were used as reference compounds. MeOH was used as the negative control. The sections were washed several times to remove cellular content which had leaked from broken cells prior to the experiments. Plates were incubated at 27 °C under light, or dark conditions. Electrical conductivity of the bathing media was measured periodically using a Horiba B-173 conductivity meter. Each treatment was triplicated. Maximum conductivity was determined by boiling ten root or coleoptile sections for 20 min.

3.4. Determination of lipid peroxidation

O. sativa seedlings were incubated in 1.25, 2.5, 5.0 and 10.0 μg ml^{-1} trichosetin **1** for 4 days. Lipid peroxidation in coleoptile samples was assayed following the procedure of Chaoui et al. (1997). Coleoptile samples were ground in 0.25% TBA in 10% TCA (10 ml g^{-1} fr. wt. of sample) with a mortar and pestle. The mixture was heated at 95 °C for 30 min, then quickly cooled in an ice-bath and centrifuged at 10,000 *g* for 10 min. The absorbance of the supernatant was measured at 532 nm and corrected for non-specific turbidity by subtracting the absorbance at 600 nm. The concentration of MDA, as the main TBA-reactive metabolite, was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer, 1965). TBA (0.25%) in 10% TCA was used as a blank.

3.5. Mitochondrial uptake of rhodamine 123

N. tabacum BY-2 cells were maintained in liquid modified MS medium (Nagata et al., 1992) at 27 °C in a gyratory shaker at 130 rpm. Four-day old cells were treated with 62.5 μg ml^{-1} trichosetin **1**. After 1 h, treated cells were stained with 6.25 μg ml^{-1} rhodamine 123 for 2 h in darkness, then washed with the culture medium twice by low-speed centrifugation, and observed under a fluorescence microscope with filter module G for rhodamine 123, or bright-field optics. MeOH was used as the negative control.

3.6. Determination of root cell viability

Germinated rice seedling was suspended in 5 ml distilled water containing either 10.0 μg ml^{-1} trichosetin **1** or 12.5 μl MeOH following the hydroponic culture of Toda et al. (1999) with minor modifications. Instead of a plastic photo slide mount and a nylon mesh, the culture apparatus was made of a piece of cheese cloth inserted between the rim and cap (bored by a hot nail head) of an Eppendorf tube. After 4 days, root tip viability was determined by staining with FDA–PI following the method of Koyama et al. (1995). Stained root tips were observed under a fluorescence microscope and photographed.

Acknowledgements

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