THE INFLUENCE OF TRIADIMENOL ON BANANA CULTIVARS BASRAI AND WILLIAMS UNDER *IN VITRO* CULTURE

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ABSTRACT

Triadimenol is a systemic fungicide with protective, curative and eradicant action. It is highly effective in controlling a variety of economically important pathogenic fungal caused diseases on plant. This study was conducted to investigate the influence of triadimenol on *in vitro* culture of banana cultivars Basrai and Williams. The effects of systemic triadimenol were tested on the shoot-tip explants. Explants were cultured on Murashige and Skoog (MS) basal solid medium supplemented with 5 mg/l 6-benzylaminopurine (BAP) or 1 mg/l indole-3-butyric acid (IBA) and different concentration of triadimenol. Results showed the viability of both banana cultivars were decreased as well as triadimenol concentration increased. Most of shoot-tip explants died at the lethal concentration (60 mg/l) of triadimenol. The shoot-tip proliferation, the number and length of lateral buds and efficiency of root system formation were decreased as the triadimenol concentrations increased. The sub-lethal concentration (50 mg/l) of triadimenol affected the photosynthetic pigments such as ChI a, ChI b and carotenoids contents and a variation in the number and stain intensity of the polypeptides compared with control ones was exhibited. Triadimenol (50 mg/l) treated shoots also showed dramatic decreasing in the Δ^5 sterols; sitosterol, stigmasterol and campesterol content. This study demonstrated that triazole fungicide triadimenol has a similar inhibitory effect on the growth and development of the two banana cultivars Basrai and Williams.

Key words: Fungicides; Proliferation; Shoot-tips; Sterol Biosynthesis; Triazoles

INTRODUCTION

Triazole derivatives represent the largest and most important group of modern systemic fungicides. They are systemically translocated in plants and have a broad spectrum of fungitoxicity. Triazole derivatives are used as fungicides and plant growth regulators in agriculture (Hartmann, 1998; Rahier & Taton, 1997). They are commonly used in agriculture because of their broad-spectrum activity against many groups of fungal diseases (Hartmann, 1998; Kaspers, 2009). Triadimenol is sterol-biosynthesis-inhibiting fungicide exhibits plant growth regulating properties (Fletcher *et al.,* 2000). It blocks gibberellin (GA) biosynthesis by inhibiting cytochrome P450-dependent mono-oxygenases which are involved in the oxidation of enr-kaurcne to entkaurenoic acid (Rademacher, 2000; Buchennauer & Rohner,1981). Besides gibberellins, the above pathway is also involved in the synthesis of abscisic acid (ABA) and cytokinins (Hartmann, 2004).

Triadimenol is highly effective in controlling a variety of economically important pathogenic fungal caused diseases on banana such as powdery mildews, sigatoka, rusts and various leaf spot diseases (Ventura *et al.,* 1994; Fidanza *et al.,* 2006). It inhibits the 14-alpha-demethylation reaction in sterols biosynthesis by interacting with the cytochrome-P450 monooxygenase of the 14-alphademethylase complex (Ancholle *et al.,* 1984; Rahier & Taton, 1997), thus cause an accumulation of 14-alpha-methyl sterols that cannot pack combine with the fatty acyl chains of the phospholipids of cell membrane. The formation of the latter is disrupted and plant growth is affected (Fletcher *et al.,* 2000; Kaspers, 2009).

Triadimenol mode of action involves demethylation of C-14 during Δ^5 sterols (sitosterol, stigmasterol and campesterol) biosynthesis, and leading to accumulation of C-14 methyl sterols (Piironen *et al.,* 2000; Rahier & Taton, 1997). The biosynthesis of these sterols is critical to the permeability and formation of plant cell membrane (Hartmann, 1998). This lack of normal sterol production slows or stops the growth of plants

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(Koller, 1987; Rademacher, 2000). Therefore, triadimenol is considered to be growth inhibiting rather than fungicidal or killing (Fidanza *et al.,* 2006). This study was conducted to investigate the triadimenol side effect on growth and development of banana cultivars 'Basrai' and 'Williams', to give some information about its effect on the host plant when used to control fungi-caused diseases.

MATERIALS AND METHODS

Plant material preparation

The experiment was carried out in Botany Department, Faculty of Science, Sohag University, Egypt. The shoot-tips were excised from two healthy banana trees grown in sandy soil farm located at Sohag Governorate. The excised shoot-tips were surface sterilized in 20% commercial Clorox solution containing 1.05% sodium hypo-chlorite added with a drop of Tween-20 for 15 min followed with in 0.1% mercuric chloride solution for 3 min. The explants were rinsed three times with sterile distilled water and transferred to sterile glass jars (250 ml) contained culture media. The medium used was Murashige and Skoog basal solid medium (Murashige & Skoog, 1962) supplemented with 30 g/l sucrose, solidified with 1% Difco Bacto agar. The pH of the medium was adjusted with KOH or HCI to 5.8 before autoclaving. All cultures were incubated in the growth chamber at the standard culture conditions of temperature (25±2ºC), light regime (16 h/d) and irradiance intensity (50 μE m^{-2} S^{-1}). All experiments were repeated twice with 25 shoot-tips for each treatment.

Effect of triadimenol on the viability of shoot-tips

Triazole fungicide, triadimenol (baytan) from Bayer AG, Lever kuse, Germany, was kindly provided by Agricultural Research Center, Ministry of Agriculture, Giza, Egypt. Triadimenol was solubilized in DMSO (Dimethylsulfoxide) before added to the sterile media.

Both 'Basrai' and 'Williams' shoot-tip explants (about 0.5 cm length) were cultured on MS solid medium (Murashige & Skoog, 1962) supplemented with 5 mg/l BAP and triadimenol at 30, 40, 50, 60 or 70 mg/l, for four weeks.

Effect of triadimenol on shoot-tip proliferation and rooting

To determine the effect of fungicide triadimenol on shoot-tip proliferation, the shoot-tips of banana cultivars were cultured on MS medium added with 5 mg/l BAP and triadimenol concentrations at 30, 40 or 50 mg/l, separately for four weeks. The effect of triadimenol on rooting was investigated by culturing Shoots approximately 3-5 cm long on MS solid medium supplemented with 1 mg/l IBA and triadimenol concentrations at l0, 20, 30, 40 or 50 mg/l for three weeks.

Chemical analysis

The photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) were determined spectrophotometrically (Spekol 11, Carl Zeiss, Jena, Germany) according to Metzner *et al.* (1965). A known fresh weight of leaves was homogenized in 85% aqueous acetone for 5 min. The homogenate was centrifuged and the supernatant was made up to known volume with 85% acetone and measured at 452, 644 and 663 nm. The concentrations chlorophyll a, chlorophyll b and carotenoids were expressed as g / ml and calculated using the following equations:

Chlorophyll a = 10.3 E 663 - 0.918 E 644

Chlorophyll b = 19.7 E 644 - 3.87 E 663

Carotenoids = 4.2 E 425.5 - (0.0264 chlorophyll a + 0.426 chlorophyll b).

Estimation of Sterol

Estimation of 4-demethyl sterols; sitosterol, stigmasterol and campesterol were carried out according to A.O.A.C (1984) using Perkin-Elmer 3920B gas chromatograph equipped with a flame ionization detector. A glass column (2 m x 2 mmID) packed with 3% (w/w) OV-Iof 80-100 mesh Gas-Chrom Q, was used isothermally at 240ºC. The carrier gas was helium at a flow rate of 40 ml/min. Injector and detector temperatures were 225 and 245ºC respectively. For quantification, 5ct-cholestane was used as internal standard, and sterols were identified by cochromatography using authentic standards

Protein Extraction:

About 1g of Leaves homogenized in liquid nitrogen, resuspended in cold solution of 10% of trichloroacetic acid (TCA) and acetone with 1% β-mercaptoethanol (β-ME), kept on -20ºC overnight. The mixture was centrifuged the next day and the pellet was resuspended in cold acetone containing 0.1% β-ME for 1 h. The pellet was dried for 30 minutes and stored at -20ºC in aliquots.

SDS-PAGE:

SDS-PAGE was performed using 15% acrylamide gel. Protein samples containing 40 μg of protein were mixed with an equal volume of buffer containing 0.125 M Tris-hydrochloric acid (Tris-HCl), pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and bromophenol blue as a tracking dye. The mixture was heated in a water bath (96ºC) for 90 s and loaded onto gel wells for

electrophoresis (Bio Rad, Protean II XI Cell). Gels were run at 18 mA per gel for 6 h at 4ºC in run buffer containing 0.025 M Tris-HCl +0.192 M glycine acid and 0.1% SDS. Protein bands were visualized by Coomassie Brillliant blue.

Statistical analysis:

The obtained data from this study were statistically analyzed using standard deviation (SD) according to the method described by Sndecor & Cochran (1980).

RESULTS AND DISCUSSION

Results showed that the sub-lethal concentration of shoot-tip explants of banana cultivars was 50 mg/l (Table 1). Most of shoot-tip explants failed to grow at the lethal concentration (60 mg/l) of triadimenol. The viability of banana cultivars 'Basrai' and 'Williams' shoot-tip explants were decreased as triadimenol concentrations increased. 'Williams' Cv was more sensitive toward triadimenol compared to 'Basrai' (Table 1). Two shoot-tip explants of Basrai were grown at the lethal concentration (60 mg/l) of triadimenol. The shoot-tips turned brown and died after subculture into triadimenol free medium. These results are in agreement with Fidanza *et al.* (2006), reported that triazole fungicide triadimenol affected many plant growth properties. The number and length of lateral buds (Table 2) were decreased as the triadimenol concentrations increased. At the sub-lethal concentration (50 mg/l) of triadimenol, the numbers of lateral buds of 'Basrai' and 'Williams' were 1.7 and 1.3, while the length of shoot buds were 0.8 cm, respectively. The number of leaves per shoot showed low variation with the increased of triadimenol concentrations. At the sub-lethal concentration (50 mg/l) of triadimenol, the leaf length of Basrai and Williams were the same (0.6). Triadimenol reduced growth of coleoptiles and primary leaves of barley seedlings (Rademacher, 2000). Extracts of triadimenol-treated shoot tissues of tomato, cotton and barley plants were contained substantially lower gibberellin-like activity than control shoots (Buchennauer & Rohner, 1981). Triadimenol interfered in gibberellin and sterol biosynthesis by inhibiting oxidative demethylation reactions (Hartmann, 2004). The plant growth retarding activity of triazole derivatives has been reported (Mercer, 1983). This might be due to the accumulation of sterol precursors in triadimenol treated wheat seedlings (Koller, 1987).

Table 1. Effect of different concentrations of triadimenol (baytan) on the viability of shoot-tip explants of banana cultivars, Basrai and Williams cultured on MS solid medium supplemented with 5 mg /l BAP for four weeks

triadimenol (mg/l)	Number of shoot-tip explants	% of The living shoot-tips (mean±SD)	
		Basrai	Williams
Control I	25	100±0.0	100±0.0
Control II	25	100±0.0	100±0.0
30	25	92±0.17	88±0.33
40	25	76±0.5	56±0.5
50	25	32±0.45	16±0.37
60	25	08±0.20	0.0±0.0
70	25	0.0±0.0	0.0±0.0

Control I = Shoot-tip explants cultured on MS solid medium + 5 mg /l BAP.
Control II = Shoot-tip explants cultured on MS solid medium + 5 mg /l BAP + 1 ml/l DMSO.

Table 2. Effect of triadimenol concentrations on proliferation of shoot-tip explants of banana cultivars, Basrai and Williams cultured on MS solid medium supplemented with 5 mg/l BAP for four weeks

Cultivar	Treatment (mg/L)	No. of buds/ Explants (cm)	Length of shoot buds (cm)	No. of leaves / shoot	Length of leaves (cm)
	Control I	4.3±0.41	1.00±0.04	3±0.33	0.72±0.03
	Control II	3.7±0.37	1.34±0.05	2±0.20	1.10±0.04
Basrai line	30	3.3±0.59	1.03±0.08	2±0.51	0.76±0.05
	40	3.3±0.51	0.90±0.04	2±0.36	0.70±0.03
	50	1.7±0.38	0.80±0.04	1±0.38	0.60±0.06
	Control I	5.3±0.51	1.14±0.05	3±0.37	0.9±0.02
	Control II	4.3±0.51	1.10±0.05	2±0.43	0.8±0.05
Williams line	30	3.7±0.29	1.10±0.03	2±0.29	0.8±0.06
	40	3.3±0.49	0.90±0.03	2±0.50	0.7±0.04
	50	1.3±0.0	0.80±0.05	1±0.48	0.6±0.03

Values are means of 25 replicates per treatment ± SD.
Control I = MS solid medium + 5 mg/l BAP.
Control II = MS solid medium + 5 mg/l BAP + 1 ml/l DMSO.

Table 3. Effect of triadimenol concentrations on the rooting of the excised shoots of banana cultivars, Basrai and Williams cultured on MS solid medium supplemented with 1mg/l IBA for three weeks

Cultivars	treatment	% of rooted shoots	No of roots /shoot	Length of roots
	Control I	100	5±0.57	8.8±1.60
	Control II	100	5±0.50	3.0±0.35
	10	100	2±0.40	0.5±0.04
Basrai line	20	60	2±0.78	0.4±0.09
	30	53	1±0.40	0.3±0.04
	40	0.0	0.0	0.0±0.00
	50	0.0	0.0	0.0±0.00
	Control	100	5±0.57	5.0±1.06
	Control II	100	8±0.50	3.0±0.70
	10	90	4±0.46	0.5±0.04
Williams line	20	40	2±0.57	0.5±0.05
	30	30	1±0.00	0.3±0.05
	40	0.0	0±0.00	0.0±0.00
	50	0.0	0±0.00	0.0±0.00

Values are means of 25 replicates per treatment ± SD.
Control I = Excised shoots cultured on MS solid medium + 1 mg/l IBA.
Control II = Excised shoots cultured on MS solid medium + 1 mg/l IBA + 1 ml/l DMSO.

Table 3 showed that the number of roots per shoot and the average length of roots were decreased as triadimenol concentration increased. The sub-lethal concentration of triadimenol for rooting of Basrai and Williams was 30 mg/l. At this concentration the percentages of shoots developed roots for Williams and Basrai were 30 and 53%, respectively. These roots seemed black and thick in their appearance Previous study by Koller (1987) also showed that root growth of wheat plant was reduced by triadimenol. Plant growth regulators (PGR) activity of triadimenol, which is discussed as a beneficial side effect was intensively investigated by Hills *et al,* (1985). Brassinosteroids are plant sterols that cause cell elongation, cell expansion, enhances gravitropism, retard abscission and promote xylem differentiation (Hartmann, 1998). The plant growth retardant effect of triadimenol may be associated with an inhibition of the biosynthetic pathway of campesterol (Hartmann, 2004). Δ^5-sterols play an important metabolic role in the cell proliferation process (Piironen *et al.,* 2000). Stigmasterol might be specifically required for cell proliferation (Hartmann, 2004).

The sub-lethal concentration (50 mg/l) of triadimenol affected ChI a, ChI b, ChI a / ChI b and carotenoids contents of Basrai and Williams cultivar (Table 4). These results correspond to those of Fletcher *et al.* (2000) who reported that triazole fungicide triadimenol effective in changing the ChI content per unit fresh weight. The surface area of some leaves of the two banana cultivars was smaller than controls. These leaves appeared intense green and thick in their phenotype. The intense greening of leaves of the treated shoots with the sub-lethal concentration (50 mg/l) of triadimenol may be attributed to the increase in ChI concentration per unit area. This observation is in agreement with

Table 4. Photosynthetic pigments content of banana cultivars, Basrai and Williams cultured on MS solid medium supplemented with 5 mg/l BAP and 50 mg/l triadimenol for four weeks.Values are means of three replicates ± SD

Variations		Pigments content (mg/g fresh weight)			
Cultivar	Treatment	Chl a	Chl b	Carotenoids	a/b
	Control I	0.28 ± 0.01	0.15 ± 0.007	0.06 ± 0.01	1.87
Basrai line	Control II	0.30 ± 0.01	0.20 ± 0.020	0.08 ± 0.01	1.50
	50 mg/l	0.03 ± 0.00	0.03 ± 0.00	0.01 ± 0.00	1.00
	Control I	0.20 ± 0.01	0.15 ± 0.02	0.06 ± 0.01	1.33
Williams line	Control II	0.23 ± 0.06	0.12 ± 0.01	0.04 ± 0.01	1.92
	50 mg/l	0.02 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	1.00

Control I = Shoot-tip explants cultured on MS solid medium +5 mg/l BAP.
Control II = Shoot-tip explants cultured on MS solid medium +5 mg/l BAP + 1 ml DMSO.

Table 5. Effect of triadimenol on sitosterol, stigmasterol and campesterol contents of banana cultivars Basrai and Williams shoots cultured on MS solid medium supplemented with 5 mg/l BAP and 50 mg/l triadimenol for four weeks

Treatment	Basrai line				Williams line			
	Sitosterol	Stigmasterol	Campesterol	Total	Sitosterol	Stigmasterol	Campesterol	Total
Control	6.7	2.5	3.9	13.1	.7.9	5.7	2.5	16.1
50 mg/L	2.3	.0.3	1.3	3.9	2.2	1.3	0.2	3.7

% was calculated as relative to the total percentage of unsaponifiable matter.
Control = MS basal medium + 5 mg/l BAP.

those reported by Kisohrekumar *et al.* (2007) who attributed the greening effect to the growth retarding activity of the fungicides. Khalil *et al.* (1990) reported that pigments probably condensed into a smaller area of the leaf, which appeared darker green than control ones.

Shoots of banana cultivars cultured in 5 mg/l BAP and 50 mg/l triadimenol showed dramatic reduction of total Δ^5-sterols contents compared with control ones (Table 5). Hartmann (2004) reported that triadimenol inhibits the synthesis of sterols possessing C-4 and C-14-methyl groups. Rahier & Taton (1997) pointed out that triazole fungicide triadimenol interacts with cytochrome P-450 obtusifoliol demethylase and thus alter sterols biosynthesis in higher plants.

Basrai and Williams cultivars exhibited a variation in the number and the stain intensity of the polypeptides bands compared to control (Table 6). In Basrai cultivar, control sample was distinguished with three polypeptides bands of molecular weights about 25, 45 and 108 kDa, while the triadimenol treated sample had nine bands of molecular weights of 26, 31, 40, 43, 46, 56, 75, 78, 86 kDa, respectively (Table 6). The variation involved in, an increased in the stain intensity and the number of polypeptides. In Williams cultivar, the treated shoots were distinguished with seven bands of molecular weights, 25, 31, 39, 48, 57, 60 and 106 kDa, respectively while control appeared six bands of molecular weights of 15, 24, 28, 46, 56 and 78 kDa. The disappearance of polypeptides of the molecular weights of 20, 24, 31, 43 and 79 kDa and decreased in the staining intensity of some bands of the treated sample compared to control one. Systemic fungicides reflect a type of particular stress conditions exhibit alteration of gene expression inducing a change in the plant

Table 6. Drawn profiles of protein patterns of the developed shoots of Basrai and Williams on MS solid medium supplemented with 5 mg/l BAP and 50 mg/l triadimenol for four week. Control = Excised shoots cultured on MS solid medium + 5 mg/l BAP + 1 ml/l DMSO

KDa	Marker	Basrai Line		Williams Line	
		Control	50 mg/l	Control	50 mg/l
13	+	+	+	+	+
15		+	+	+	0
24	+	+	+	+	0
25		+	0	0	+
26		0	+	0	0
28		0	0	+	0
31		0	+	0	+
39		0	0	0	+
40		0	+	0	0
43		0	+	0	0
45		+	0	0	0
46		0	+	+	0
48		0	0	0	+
55	+	0	0	0	0
56		0	+	+	0
57	+	0	0	0	+
60		0	0	0	+
75	+	0	0	0	0
78		0	+	+	0
86		0	+	0	0
106		0	0	0	+
108	+	+	0	0	0

metabolism resulting in an alteration in the protein synthesis which may vary according to the phenotype of plant (Wetzstein *et al.,* 2002). The changes of protein profile are a reflection to the biochemical events that reflect various biochemical responses of plants to the influence of triazole fungicide on the shoot organogenesis (Schrick *et al.,* 2000). This study revealed that, the systemic triazole fungicide triadimenol might had an inhibitory effect on the growth and development of banana cultivars and its inhibitory effect was similar in both two desert banana cultivar, Basrai and Williams.

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