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Comparative Metabolite Profiling of the Insecticide Thiamethoxam in Plant and Cell Suspension Culture of Tomato

Rajib Karmakar,[†] Ramcharan Bhattacharya,[‡] and Gita Kulshrestha*[,†]

[†]Division of Agricultural Chemicals, Indian Agricultural Research Institute, New Delhi-110012, India, and [‡]National Research Centre on Plant Biotechnology, Indian Agricultural Research Institute, New Delhi-110012, India

The metabolism of thiamethoxam [(*EZ*)-3-(2-chloro-1,3-thiazol-5-yl-methyl)-5-methyl-1,3,5-oxadiazinan-4-ylidene (nitro) amine] was investigated in whole plant, callus, and heterotrophic cell suspension culture of aseptically and field grown tomato (*Lycopersicon esculentum* Mill.) plants. The structure of the metabolites was elucidated by chromatographic (HPLC) and spectroscopic (IR, NMR, and MS) methods. Thiamethoxam metabolism proceeded by the formation of a urea derivative, a nitroso product, and nitro guanidine. Both urea and nitro guanidine metabolites further degraded in plants, and a mechanism has been proposed. In the plant, organ-specific differences in thiamethoxam metabolism were observed. Only one metabolite was formed in whole plant against four in callus and eight metabolites in cell suspension culture under aseptic conditions. Out of six metabolites of thiamethoxam in tomato fruits in field conditions, five were similar to those formed in the cell suspension culture. In the cell suspension culture, thiamethoxam degraded to maximum metabolites within 72 h, whereas in plants, such extensive conversion could only be observed after 10 days.

KEYWORDS: Tomato; *Lycopersicon esculentum* Mill.; pesticide metabolism; neonicotinoid insecticide; thiamethoxam; cell suspension culture; biotransformation; metabolites; IR; NMR; LC-MS; chemical characterization

INTRODUCTION

Thiamethoxam, a neonicotinoid insecticide, is applied to a wide variety of crops including cucurbits and fruiting vegetables such as tomato (*Lycopersicon esculentum* Mill) to protect from insect pests such as aphids, whiteflies, plant hoppers, and some lepidopteran pest species (*1*). It acts as an agonist of the nicotinic acetylcholine receptor, affecting the synapses in the insect's central nervous system (*2*, *3*). It is used primarily as a systemic insecticide undergoing absorption and translocation for crop protection.

In the process of distribution, a pesticide encounters several enzymes and is transformed through oxidation, reduction, or hydrolysis to generally produce a more polar and usually a less toxic product than the parent compound. Plant metabolism studies are thus important in determining the tolerance and efficacy of a pesticide. Thiamethoxam metabolism has been studied in mice (*4*) and in a few plants such as cotton and spinach (*5*). Diversity in metabolic pathway depends on the chemical structure of the pesticide, plant species, and the metabolic enzymes. The same pesticide in different plant species may degrade to different metabolic products (*6*). The type and toxicology of the transformation products of the pesticides are important in deciding food safety to the consumer. Identification of the metabolites is not only of academic interest but is also an important aspect of registration for use and assignment of tolerance values. When applied to cotton plants, thiamethoxam was quickly metabolized, with clothianidin being the predominant neonicotinoid in plants briefly after application, as indicated by LC-MS/MS analyses (*7*). Interestingly, the N-desmethylated derivative of thiamethoxam, *N*-desmethyl thiamethoxam, was not significantly produced in cotton plants, although it was often mentioned as a possible metabolite, being nearly as active as imidacloprid.

Plant tissue cultures are powerful tools for metabolism studies. Culture conditions which mimic conditions of whole plants can be selected (*8*). Culture variables that affect metabolism are medium composition, age of tissue cultures, concentration of test chemical, and the source of plant tissue (*9*). The type of culture, such as suspension cultures, callus tissue cultures, and differentiated tissue or organ cultures also influence the type of metabolites obtained (*10*−*13*). It is generally accepted that heterotrophically cultured plant cells metabolize pesticides in a manner similar to that of whole plants but differ in the quality and quantity of metabolites formed because in vitro cultures essentially have no barriers to pesticide penetration or translocation, and little or no bound-pesticide residues are formed (*14*).

This study describes the establishment of cell suspension cultures from friable callus, which was obtained from tomato (var. Pusa Ruby) seedling stems. Using different concentrations and combinations of kinetin, benzylaminopurine (BAP), auxin, and 2,4-dichlorophenoxyacetic acid (2,4-D), the growth patterns

*To whom correspondence to be addressed. Tel: 91+11+25972191. E-mail: gitakul@rediffmail.com.

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of the cultures were examined during a range of culture durations. The optimized culture conditions obtained from the present study were then used in the tissue culture experiments to identify thiamethoxam metabolites. The products were chemically characterized by cochromatography with authentic standards prepared in the laboratory using HPLC-DAD and by LC-MS. The metabolites produced in aseptic conditions were compared with those formed in tomato fruits under field experiments. This is the first report of thiamethoxam metabolism in tomato using cell suspension culture technique.

MATERIALS AND METHODS

Chemicals. All of the chemicals and solvents were of analytical grade. HPLC grade solvents and water were degassed, by vacuum filtration, prior to use. Tomato seeds (var. Pusa Ruby) were procured from National Seed Corporation (NSC) of India, New Delhi.

Thiamethoxam and Its Authentic Degradation Products. Thiamethoxam (Analytical grade) and Actara (25 WDG) were obtained from M/S Syngenta India Ltd. Purity of the analytical grade insecticide was assessed by IR, NMR, and mass spectroscopy. IR $\nu_{max}{}^{KBr}$ (cm^{-1}): 1597 and 1396 (-NO_2 str). ^1H- NMR ($CDCl_3$ + DMSO-d_6) δ: 2.95 (s, 3H-nCH_3); 4.72 (s, 2H, $-CH_2$-N<); 4.8−5.0 (d, $J = 2.5H_z$, 4H, 2×O−CH_2-N) and 7.55 (s, 1H, −CH=). The mass spectrum shows m/z 291.8 (M^+), 245.8 (M^+-NO_2), 211.0 (M^+-$ClNO_2$), and 132.0 (M^+-$C_4N_4O_3H_7$). A stock solution of thiamethoxam (1000 μg mL^{-1}) prepared in HPLC grade water and sterilized by passing through a bacterial filter was used in all of the experiments.

Structures of the two degradation products of thiamethoxam prepared in the laboratory (*15*) were elucidated by HPLC and spectroscopic techniques. Compound II (*tR* of 1.31 min) was a crystalline, colorless solid (mp. 120−121 °C). ^1H NMR ($CDCl_3$ + DMSO-d_6) δ: 4.51 (d, $J = 7$ Hz, 2H, OH D_2O exchange S, CH_2NH_2); 6.48 (6s, 2H, exchangeable with D_2O, -NH_2); 7.38 (s, 1H, =CH−) and IR $\nu_{max}{}^{KBr}$(cm^{-1}) 3150 (−NH_2). The product was characterized as 2-chloro-5-thiazolemethanamine. Compound IX (*tR* of 8.31 min) was crystallized as a colorless crystalline product (mp 110 °C). The NMR and mass fragmentation pattern of the compound were as follows. ^1H NMR ($CDCl_3$ + DMSO-d_6) δ: 2.90 (s, 3H, CH_3); 4.55 (s, 2H, −CH_2); 4.62−4.82 (d, $J = 2$−3 Hz, 4H, -N−CH_2-O−CH_2-N); 7.38 (s, 1H, =CH−) and MS (m/z): 247.5 (M^+); 213 (M^+-Cl); 183 (213-NH_3); 132 (M^+-$C_4N_2OH_7$). The structure was established as 3-(2-chloro-1,3-thiazol-5-ylmethyl)-5-methyl-1,3,5-oxadiazin-4-one, a urea derivative.

Treatment of Plants under Aseptic Conditions. Seeds of tomato cultivars were surface sterilized by a solution of 0.1% $HgCl_2$ and 0.1% sodium dodecyl sulfate for 5 min followed by repeated washes in sterile water. About 5−7 seeds were sown in half strength minimal salt (MS) medium (*16*) in a jam bottle (12.5 cm height × 6.5 cm diameter) under completely aseptic condition. The incubation conditions for germination and in vitro culture were 25 ± 1 °C and 16 h photoperiod of approximately 28 m Em^{-2} s^{-1}. For application to aseptically grown whole plants of three weeks, thiamethoxam was sprayed at a dose 100 times the recommended dose (140 g ha^{-1}). After 30 days of application, plants were taken out for extraction. Each sample consisted of a minimum of three independent plants.

For generation of friable callus, hypocotyls of 10 day old tomato seedlings, grown as described above, were cut into pieces (approximately 5 mm) and placed onto the MS medium supplemented with 3% sucrose. Using different concentrations and combinations of the phytohormones such as cytokinin, auxin, and kinetin, we examined the growth patterns of the cultures during a range of culture durations. The optimized culture conditions obtained from the study were then used in further experiments. Seven combinations of cytokinins and auxin concentrations were used to check the growth and nature of the callus. The hormone combinations were variable levels of either 2, 4-D or NAA and BAP or IAA and kinetin, which are designated as A, B, C, D, E, F, and G (**Table 1**). The plates were incubated for 21 days in the culture room maintained at 25 ± 1 °C and a 16 h photoperiod of approximately 28 mEm^{-2} s^{-1} light. For application to callus, fully grown calluses (about 6 weeks grown) were dipped in a filter sterilized thiamethoxam standard solution. These fortified calluses were replaced in a Petri dish containing the desired media (as described earlier to develop friable callus) for its growth. Application was carried out in triplicate. Each petridish containing three calluses is considered as a replicate. The calluses were harvested after 15 days of insecticide application for analysis.

For cell suspension culture, well grown friable calluses generated from hypocotyls were transferred in 50 mL MS liquid medium containing 3% sucrose and supplemented with hormone combinations of B in Erlenmeyer flasks. The flasks were shaken for another 3 weeks at 125 rpm speed in a horizontal shaker placed inside the culture room. To the in vitro grown cell suspension culture, thiamethoxam was applied at 100 times the normal recommended dose. Each application was carried out in triplicate (each conical flask is considered as a replicate). Cells were harvested after 3 days of application and identification of metabolites in the cell suspension culture undertaken.

Sample Preparation and Extraction. After the desired incubation time, whole plants and calluses were frozen in liquid nitrogen and ground using a mortar and pestle. The crushed portions of the whole plants and calluses were shaken with acetone for 20 min in a conventional shaker, filtered through a Buchner funnel using vacuum, and solvent evaporated on a rotary vacuum evaporator to dryness. The residues were dissolved in acetonitrile for HPLC analysis.

After the desired incubation period, the cell suspension culture in the Erlenmeyer flask was filtered through Whatman No. 42 filter paper. The filtrate was partitioned with dichloromethane (3 × 20 mL), and the combined organic phase after drying over anhydrous sodium sulfate was concentrated on a rotary vacuum evaporator to dryness. The residue was dissolved in acetonitrile for further analysis by HPLC-DAD and LC-MS.

Treatment of Crops in the Field and Analysis of Tomato Fruits. A tomato crop was raised in the experimental field of Indian Agricultural Research Institute (IARI), New Delhi, India using normal agronomic practices (*17*). For metabolic studies, thiamethoxam was sprayed at 10 times the recommended (0.140 kg ha^{-1}) dose on 10 selected plants in a row in a plot in the field at fruit setting stage. The plants were labeled and covered with nylon nets to protect the fruits from birds and human interferences.

Tomato fruit samples were collected 10 days after insecticide spraying. The chopped sample (100 g) was blended in a warring blender with acetone (100 mL) and the contents filtered through a Buchner funnel using vacuum. Extraction was repeated twice more (2 × 50 mL), and filtrates were combined. The solvent was evaporated using a rotary vacuum evaporator (35−40 °C), and the concentrate was transferred to a separatory funnel. The contents were diluted with saline water (15% NaCl, 100 mL) and partitioned thrice with hexane (3 × 20 mL), and the hexane layer was discarded. The aqueous layer was partitioned thrice with dichloromethane (3 × 30 mL), the combined organic phase was dehydrated, and the solvent was evaporated to almost dryness. The residue was dissolved in acetonitrile, made up to volume and filtered through an HP filter (13 mm × 0.45 μm) prior to HPLC analysis.

Analysis of Thiamethoxam and Metabolites. HPLC-DAD and LC-MS conditions were the same as those in our previous studies (*15*). All retention times (*tR*) in this article are both for the HPLC-DAD and LC-MS conditions. Metabolites of the parent neonicotinoid were qualitatively analyzed on the basis of the absorbances at 254 nm.

A Hewlett-Packard high performance liquid chromatographic (HPLC) instrument (series 1100) equipped with a degasser, quaternary pump, photodiode array detector connected with a rheodyne injection system, and a computer (Model Vectra) was used for the analysis of the insecticide and allied products. The stationary phase consisted of a Lichrosphere RP-18 packed stainless steel column (250 mm × 4 mm i.d.). The mobile phase was an acetonitrile/water gradient maintained at a flow rate of 1.0 mL min^{-1}. Gradient programming starting from 85% water/acetonitrile to 60% water/acetonitrile within 10 min followed by 10% water/acetonitrile was used for the best resolution and separation of parent compound and degradation products from peaks of various coextractives in plant extracts. A 20 μL aliquot of the sample volume was injected each time and chromatograms recorded in a Windows 95 NT based HP Chemstation program. Mass spectral analysis was performed using an Agilent 1100 series LC-MS instrument with ESI (+) mode. LC conditions were similar to those in the HPLC.

RESULTS

Chromatography and Structural Assignments. Authentic samples of metabolites were prepared and characterized by spectroscopic techniques. Different metabolites formed in plants were characterized either by cochromatography (HPLC) with authentic samples or LC-MS techniques. The identified compounds have been listed in **Table 2** with their *tR* and techniques used for characterization.

Metabolite in Whole Plant under Aseptic Conditions. The HPLC chromatogram of the whole plant extract showed one peak at *tR* of 7.34 min. The LC-MS analysis of this compound (**Table 3**) gave a mass fragmentation pattern as 249.5 (M^+, 100); 205.5 (M^+-N_2O, 7); 168.0 (M^+-Cl and $-NO_2$, 45); 132.5 (M^+-$C_2H_5N_4O_2$, 90) and was assigned the structure 1-(2-chloro-1,3-thiazol-5-yl)-methyl-3-methyl nitro guanidine (VIII).

Table 1. Various Hormonal Combinations Supplemented in MS Media with 3% Sucrose to Optimize Callus and Cell Suspension Culture of Tomato Cultivar Pusa Ruby

hormonal preparation	concentration combination
A	0.1 ppm 2, 4-D
B	0.5 ppm 2, 4-D
C	0.5 ppm NAA + 2.0 ppm BAP
D	0.1 ppm 2, 4-D + 2.0 ppm BAP
E	0.5 ppm NAA + 1.0 ppm BAP
F	1.0 ppm BAP + 2.0 ppm NAA
G	4.0 ppm kinetin + 4.0 ppm IAA

Metabolite in Callus under Sterilized Conditions. HPLC analyses of the callus extract showed four peaks at retention times of 1.36, 7.50, 10.26, and 11.67 min (**Figure 1**) in addition to the peak of the parent compound thiamethoxam. LC-MS analysis of these peaks gave mass fragmentation patterns, and the structures of these metabolites were characterized as chloro thiazolemethanamine (II), nitro guanidine derivative (VIII), thiocyanate derivative (XII), and 6-hydroxy thiamethoxam (XIII), as described in **Table 3** and **Figure 2**.

Metabolites in Cell Suspension Culture. Among the hormone combinations (**Table 1**) used for callus generation, higher callus growth was achieved with B, C, or G. For cell suspension culture, the same media composition with hormone combination B was used in liquid media. After 3 days of thiamethoxam application, the cells were harvested by filtration through Whatman filter paper. HPLC analysis of the cell extract showed eight peaks (**Figure 1**) besides that of parent compound. Metabolites at *tR* of 1.31 min and 8.50 min were identified by cochromatography with authentic degradation products prepared in the laboratory. These metabolites were identified as chloro thiazolemethanamine (II) and 3-(2-chloro-1,3-thiazol-5-ylmethyl)-5-methyl-1,3,5-oxadiazin-4-one (IX), respectively. Chemical characterization of other metabolites was done by mass fragmentation pattern on LC-MS. These were identified as nitroso derivative (III, M^+ 261.8), 2-hydroxy thiazole derivative (V, M^+ 229.0), ether derivative (VII, M^+ 221.8),

Table 2. Characterization of Various Degradation Products of Thiamethoxam

common name with their assigned number in bracket	physical state and melting point (°C)	HPLC retention time (min)	characterization method
thiamethoxam (I)	crystal (pure), 139	6.15	IR, NMR, LC-MS
chloro thiazolemethanamine (II)	crystal (pure), 121	1.31	IR, NMR, LC-MS
nitroso derivatives (III)	liquid (mixture)	2.35	LC-MS
hydroxy derivative (V)	liquid (mixture)	3.69	LC-MS
guanidine derivative (VI)	liquid (mixture)	4.02	LC-MS
ether derivative (VII)	liquid (mixture)	5.37	LC-MS
nitro guanidine derivative (VIII)	liquid (mixture)	7.36	LC-MS
thiamethoxam urea (IX)	crystal (pure), 110	8.72	NMR, LC-MS
methyl urea derivative (X)	liquid (mixture)	9.07	LC-MS
thiocyanate derivative (XII)	liquid (mixture)	10.31	LC-MS
6-hydroxy derivative (XIII)	liquid (mixture)	11.67	LC-MS

Table 3. IUPAC Chemical Names and Mass Fragmentation Pattern with the Relative Intensity of Various Metabolites of Thiamethoxam Formed in Tomatoes as Identified by LC-MS

IUPAC name based on chemical structure and assigned number in parentheses	presence of −Cl	mass fragmentation (relative Intensity)
thiamethoxam, (I)	M + 2	291.8 (M^+), 245.8 (M^+-NO_2), 211.0 (M^+-$ClNO_2$) and 132.0 (M^+-$C_4N_4O_3H_7$)
2-chloro-5-thiazolemethanamine, (II)	identified by cochromatography with the authentic degradation products	
3-(2-chloro-1,3-thiazol-5-ylmethyl)-5-methyl-1,3,5-oxadiazinan-4-ylidene (nitroso) amine, (III)	M + 2	245.5 (M^+-O, 17); 172.5 (M^+-$C_2H_5N_2O_2$, 8); 115.0 (M^+-NO and C_3NClS, 22)
3-(2-hydroxy-1,3-thiazol-5-ylmethyl)-5-methyl-1,3,5-oxadiazinan-4-ylidene (nitro) amine, (V)		187 (M^+-C_2H_2O, 7); 128 (213-$C4H_7O_2N$, 10); 116 (M^+-C_4H_4NOS, 100)
3-(2-chloro-1,3-thiazol-5-ylmethyl)-amino methyl amino ethyl ether, (VII)	M + 2	206 (M^+-CH_3, 6); 177.8 (M^+-C_2H_6N, 13); 142 (M^+-Cl, 8); 119 (M^+-$C_4N_2H_{11}O$, 15)
1-(2-chloro-1,3-thiazol-5-yl)-methyl-3-methyl nitro guanidine, (VIII)	M + 2	205.5 (M^+-N_2O, 7); 168.0 (M^+-Cl and $-NO_2$, 45); 132.5 (M^+-$C_2H_5N_4O_2$, 90)
3-(2-chloro-1,3-thiazol-5-ylmethyl)-5-methyl-1,3,5-oxadiazin-4-one, (IX)	identified by cochromatography with the authentic degradation products	
N-methyl-*N'*-(2-chloro-1,3-thiazol)-5-yl-methyl-urea, (X)	M + 2	171 (M^+-Cl^-, 100); 147 (M^+-C_2H_4NO, 30); 132.5 (147-NH, 48)
2-chloro-1,3-thiazolyl-5-yl-methyl thiocyanate, (XII)	M + 2	139 (M^+-Cl^-, 100); 132.5 (M^+-NCO, 48)
3-(2-chloro-1,3-thiazol-5-ylmethyl)-6-hydroxy-5-methyl-1,3,5-oxadiazinan-4-ylidene (nitro) amine, (XIII)	M + 2	229.5 (M^+-Cl, 2); 205.5 (M^+-$C_2H_5O_2$, 4); 176.5 (M^+-$C_3H_7NO_2$, 3).

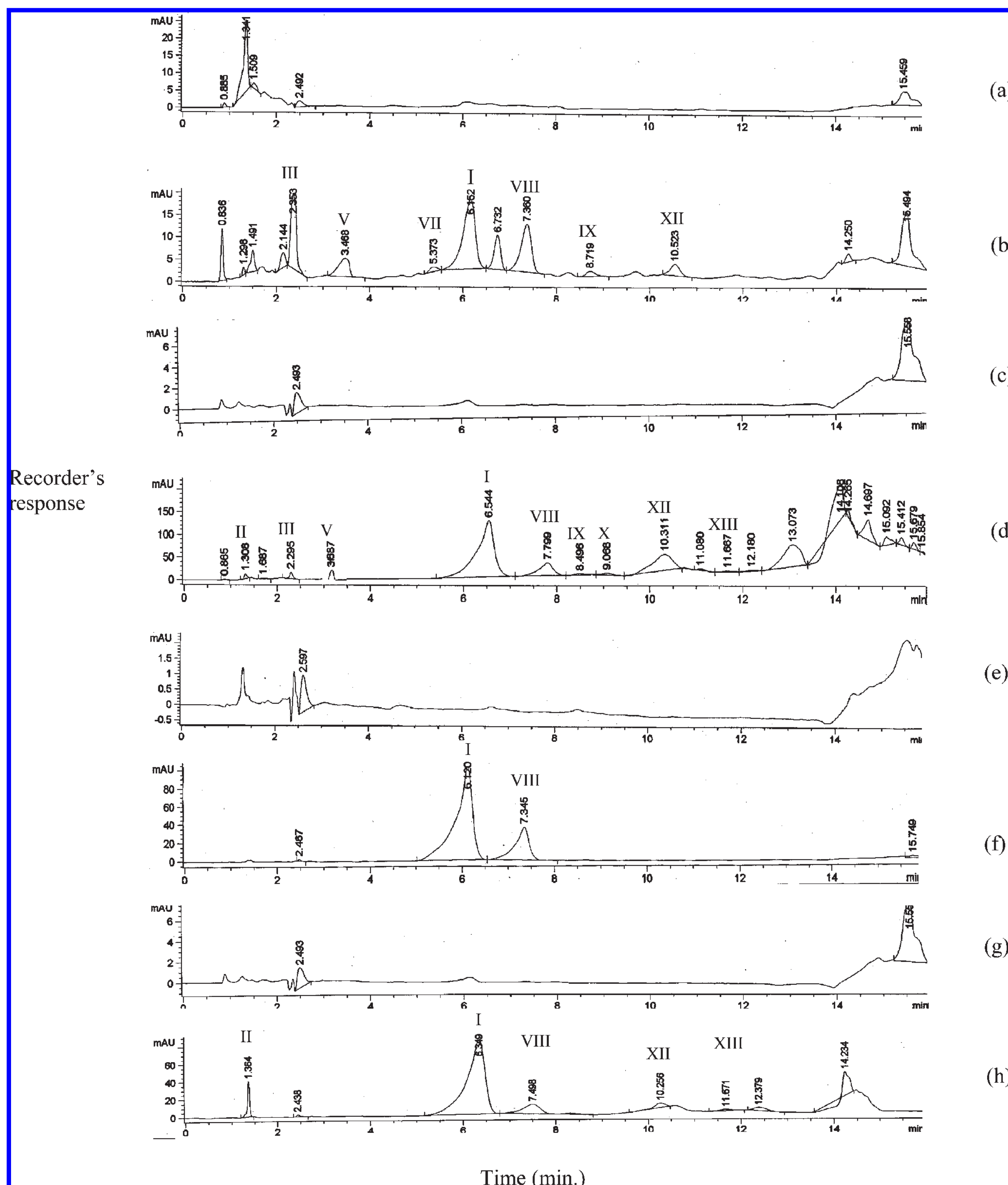

Figure 1. HPLC chromatograms of metabolites formed under different conditions. (**a**) Untreated fruit under field conditions after 10 days. (**b**) Treated fruit under field conditions after 10 days. (**c**) Untreated cell suspension culture after 3 days. (**d**) Treated cell suspension culture after 3 days. (**e**) Untreated in vitro grown whole plant at 30 days. (**f**) Treated in vitro grown whole plant at 30 days. (**g**) Untreated callus at 30 days. (**h**) Treated callus at 30 days.

nitroguanidine compound (VIII, M^+ 249.5), methyl urea compound (X, M^+ 205.5), thiocynate derivative (XII, M^+ 174.5), and 6-hydroxy derivative (XIII, M^+ 264.8), as shown in **Figure 2**.

Metabolites in Tomato Fruits. Extracts of tomato fruits were analyzed by HPLC. The chromatogram showed the presence of six peaks at retention times (*tR*) 2.35, 3.47, 5.37, 7.36, 8.72, and 10.52 min. (**Figure 1**). Desired peaks were identified by cochromatography on HPLC (**Table 2**) and/or on the basis of their mass fragmentation patterns by LC-MS (**Table 3**). Peak at *tR* of 2.35 min gave a mass fragmentation pattern as 261.8 (M^+, 100); 245.5 (M^+-O, 17); 172.5 (M^+-$C_2H_5N_2O_2$, 8); 115.0 (M^+-NO and C_3NClS, 22) and was identified as 3-(2-chloro-1,3-thiazol-5-ylmethyl)-5-methyl-1,3,5-oxadiazinan-4-ylidene (nitroso) amine (III). Likewise, the peak at *tR* of 3.47 min was characterized as the hydroxyl derivative (V) and at *tR* of 5.37 min as ether derivative (VII). The peak at *tR* of 7.36 min showed the mass fragmentation pattern 249.5 (M^+, 100); 205.5 (M^+-N_2O, 7); 168.0 (M^+-Cl and − NO_2, 45); 132.5 (M^+-$C_2H_5N_4O_2$, 90), and the structure was derived as 1-(2-chloro-1,3-thiazol-5-yl)-methyl-3-methyl nitro guanidine (VIII). The peaks at *tR*s 8.72

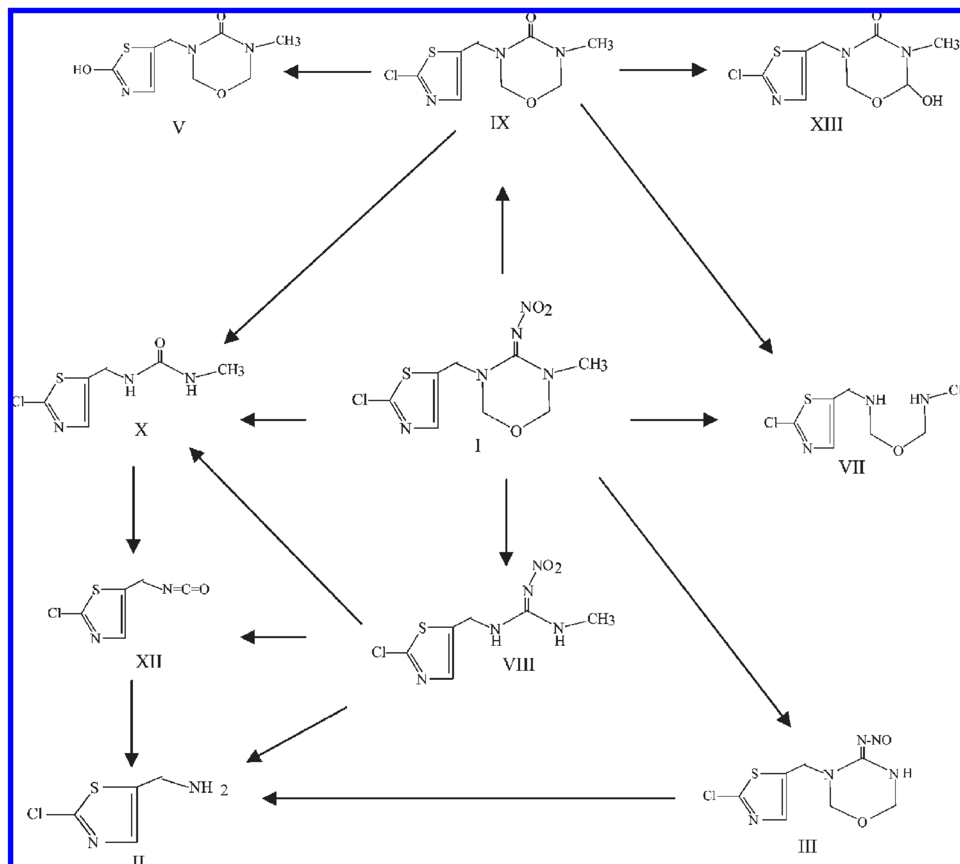

Figure 2. Postulated metabolic mechanism of thiamethoxam (I) in tomato plants. Metabolites detected in tomato fruits in field trial, III, V, VII, VIII, IX, and XII; whole plant under aseptic conditions, VIII; callus under aseptic conditions, II, VIII, XII, and XIII; cell suspension culture, II, III, V, VIII, IX, X, XII, and XIII.

and 10.52 min were characterized as compounds IX and XII, respectively.

DISCUSSION

Thiamethoxam, a relatively new member of the neonoicotinoid family has been effectively employed as both a soil and foliar insecticide in various vegetable crops including tomatoes. Being systemic in nature, this insecticide is readily absorbed within plants and gets translocated to reach the target sites. During the process, the parent compound gets degraded to various metabolites influenced by the plant enzymes. A comparative metabolite profiling of thiamethoxam formed in a cell suspension culture of tomato plants and fruits in field conditions has been presented.

Metabolite Profiling in Cell Suspension Culture. The actively growing meristematic cells in the cell suspension culture demonstrated the degradation of thiamethoxam into an array of metabolites. Within 72 h of application, thiamethoxam metabolized to eight (II, III, V, VIII, IX, X, XII, and XIII) products in the cell suspension culture (**Figure 1**). A similar study on the metabolism of imidacloprid in cell suspension cultures of *Nicotiana tabacum* (*18*) reported the formation of two metabolites within a short time of 48 h. In another study, ^{14}C-labeled metamitron, a triazinone herbicide, transformed into two metabolites by transgenic tobacco cell cultures within 48 h of incubation, and the metabolites were identified by GC-MS and LC-MS (*19*). It was concluded that cell suspension culture was an ideal method for metabolite profiling under asceptic conditions as only one metabolite (nito guanidine, VIII) was identified at the aseptically grown whole plant level and four metabolites in the case of callus culture (II, VIII, XII, and XIII).

Metabolites in Tomato Fruits under Field Conditions. The studies on field treatment of thiamethoxam revealed the formation of six metabolites in tomato fruits in 10 days. Among these metabolites (III, V, VII, VIII, IX, and XII), five were similar to those produced in the cell suspension culture. However, one additional metabolite (VII, ether derivative) identified in fruits was not detected in any of the in vitro studies. This could be due to the specific enzyme present in tomato fruits. It was thus concluded that cell suspension culture technique was at par with the field study for identification of plant metabolites without any hazardous implications.

Mechanism of Thiamethoxam Metabolism in Tomato Plants. Despite the different types of experiments, a rather uniform picture of the metabolic behavior of thiamethoxam in tomato plants was found to consist of three principal metabolic pathways (**Figure 2**). As depicted in **Figure 2**, oxidation of oxadizinan ring produced a urea metabolite (IX), which further metabolized by hydrolysis in two ways. Dechlorohydroxylation of the thiazole group led to the formation of hydroxy thiazole urea derivative (V), while oxadiazinan ring hydroxylation formed 6-hydroxy oxadiazinon (XIII). Second, a nitro-group reduction takes place in the insecticide to form the nitroso compound. Third, ring cleavage at the ether linkage of the oxadiazinan ring of thiamethoxam (I) metabolized to nitro guanidine (VIII), the product already developed and marketed as neonicotinoid insecticide clothinidin. Thiamethoxam and clothianidin differ structurally only in the thiamethoxam oxadiazinane CH_2OCH_2 moiety, which is metabolically labile on methylene hydroxylation leading to partial conversion of thiamethoxam to clothianidin. Similar ring cleavage at the ether linkage of the oxadiazinon (IX) formed

Table 4. Thiamethoxam Metabolites Identified in Tomatoes under Field and Different in Vitro Conditions

conditions	metabolites formed								
	II	III	V	VII	VIII	IX	X	XII	XIII
			In Vivo						
tomato fruits in field		Y	Y	Y	Y	Y		Y	
			In Vitro						
cell suspension culture	Y	Y	Y		Y	Y	Y	Y	Y
sterilized whole plant					Y				
sterilized callus	Y				Y			Y	Y

methylene urea derivative (X), which further degraded to thiocyanate (XII) and then to aminomethyl chlorothiazole (II). The cleavage of the urea moiety of the oxadiazinon ring in compound (IX) formed ether compound VII. Formation of these metabolites of thiamethoxam has been also reported in spinach (*5*). Thus, thiamethoxam yielded two sets of metabolites, one with the oxadiazine ring intact and the other the ring-opened clothinidin derivatives. It is of concern that thiamethoxam is transformed to clothianidin in tomato plants, although it further undergoes degradation. This is an important finding, which may be kept in mind when formulating management strategies based around this neonicotinoid.

In different experiments, significant qualitative differences were evident in the formation of thiamethoxam metabolic products in tomato plants (**Table 4**). Some of these differences could be attributed to the relatively shorter residence time of the pesticide. The cell suspension culture method is simple, sensitive, and rapid for metabolite identification. This specialized method could provide information in just 1/3rd the time that is required in field study. Besides being rapid, this method provides advantages such as prediction of metabolites that are likely to be present before initiation of an in vivo study, generation of metabolites in sufficient quantities for identification, and detection of intermediate metabolites, which may provide insight into the metabolic pathway. Another advantage of plant cell cultures is that high pesticide concentrations (i.e., concentrations that exceed the pesticide water solubility) can be used because the metabolite produced by the cells shifts the equilibrium, allowing more pesticide to dissolve in the culture medium (*20*). It needs to be mentioned that the findings of the metabolism studies reflect worst-case scenarios and that under practical field conditions lower amounts of metabolites and a more rapid degradation are envisaged. This is due to UV photolysis (*21*) and weather conditions being only of negligible importance in laboratory controlled conditions. Various physiological, environmental, and edaphic factors as well as dose influence the metabolism and ultimate disposition of the pesticide and its metabolic products in plants (*22*).

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