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Effect of PVY Viral Infection on Alkaloid Contents of Cultivated Medicinal Plants

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Abstract: Physiological studies in relation to development and growth of medicinal plants *Datura metal* are rather restricted. This study analysed *Potato virus* Y, *Potyvirus* affecting plant growth and alkaloid formation in *D.metal*. Quantitative data on branch and leaf formation, stem growth, fresh and dry weights were systematically collected at three months. It was found that the branching rate of PVY infected plant was higher than healthy ones. On the other hand, the plant height, number and area of leaves, fresh weight and dry mater were decreased in PVY infected plants compared with the increase in healthy ones. The PVY infection led to a decrease in total alkaloids (calculated as hyoscyamine) in different origin of infected plant. On the contrary, the virus infection leads to an increase in the endogenous salycilic acid in infected leaves. Twenty one alkaloids were identified in the leaves of *D.metal* by gas chromatography and mass spectrometry. Fifteen of them were found in healthy leaves and twelve in the infected ones. Further more, a new tropane/ester was tentatively identified as 3-(3-formyloxytropoyloxy) tropane on basis of the mass spectral fragmentation. Hyoscyamine was the main alkaloid in leaves.

Key words: *Datura metal*, Gas chromatography, mass spectra, hyoscyamine, salycilic acid, alkaloid, PVY.

INTRODUCTION

Many higher plants accumulate extractable organic substances in quantities sufficient to be economically useful as chemical feed stocks or raw materials for various scientific, technological and commercial applications. Natural substances are employed, either directly, by a large number of industries and natural plant products (phytochemicals) figure prominently in several of these[19]. Economically important plants serve as sources of industrial oils, resins, tannins, saponins, natural rubber, gums, waxes, dyes, pharmaceuticals,,and many specialty products[19, 23]. In higher plants such compounds are often concentrated in seeds and vegetative storage organs and are needed for physiological development because of their role in basic cell metabolism. *Datura ceratocaula* (Solanacea) is an aquatic, hollow stemmed, prostrate, creeping plant known by the Mexicas as the narcotic tornaloca or maddening plant. This species is the connecting link between herbaceous daturas and rugmensias[11, 17]. Only a few alkaloids have been reported for *D. ceratocaula*[3, 14, 11]. Knowledge of the complete alkaloid pattern is of interest not only phytochemically, but also in relation to aspects of alkaloid biogenesis metabolism and application in the plant biotechnology. Recent investigations of genus Datura and Brugmansia with modern analytical methods namely Gas chromatography and mass spectrometry, demonstrated that tropane alkaloid containing plants generally have a large number of alkaloid, which are not detected by other methods[24, 7, 1].

A new study on the effect of viral infection on alkaloid content, the most important group of secondary metabolites, of cultivated medicinal plants[9] is demonstrated in this work on the selected plant *Datura metal*.

MATERIALS AND METHODS

The seeds of *D.metal.L* were kindly provided by the National Organization of Drug Central and Research, Giza, Egypt.

Greenhouse pot experiment was carried out using fertile clay loam soil collected from the farm of the Faculty of Agriculture, Ain shams University.

The soil was distributed in earthenware pots (30 cm in diameter). For each treatment then pots were prepared. Five seeds of *D.metal.L* were planted in each pot. The plants were thinned two months after sowing and three plants left per pot. Morphological characteristics and active constituent of plants were determined after six months of cultivation.

Morphological characteristics recorded were, rate of branching, number of leaves plant $^{-1}$, plant height, total fresh weight and dry weight.

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Determination of Alkaloids: The active constituent was determined after four months of cultivation using the method described by the British Pharmacopocia[8]. The active constituent was expressed as gram hyoscyamine per 100g of dry matter. Each mL of H_2SO_4 is equivalent to 0.01447 g of alkaloids calculated as hyoscyamine.

Quantification of Endogenous Salysilic Acid (SA): SA was measured in leaves of infected *D.metal* plants with PVY and healthy ones. Samples were prepared as described by Raskin *et al*.[21] with one modification that total free and conjugated SA were measured directly using β- glucosidase enzyme. One gram of frozen tissue was ground in 3 mL of 90% methanol and centrifuged at 6000 g for 15 min. The pellet was back extracted with 3 mL of 99.5% methanol and centrifuged. Methanol extracts were combined and centrifuged at 1500 to 2000 g for 1 min and dried at 40°C under vacuum using rotary evaporator (Heid alph.). Dried extracts were then resuspended in 3 mL of water at 80°C and an equal volume of 0.2 M sodium acetate buffer (pH4.5) containing 0.1 mg/mL β- glucosidase (22unit/mg, Sigma). This mixture was incubated at 7° C overnight. After digestion, mixture was acidified to pH 1.0 to 1.5 with HCl. SA was extracted for quantification by HPLC into volumes of cyclopentan/ ethylacetate/ isopropanol 50:50:1. The organic extract was dried by liquid nitrogen and analyzed by HPLC (SHIMADZO RF-10AXL.Flurescence) as described previously[21]. Twenty five microliter samples were injected into Dyanax 60 A8 m guard column (46mmx 1.5cm) linked to 40°C. SA was separated with column with 23% v/v methanol in 20 mM sodium acetate buffer (pH 5.0) at a flow rate of 1.5 mL min^{-1}. SA level was determined using standard curve.

Alkaloid Extraction: Plant samples were dried at 50°C and cerate in 3% H2So4 for 24 h at room temperature. The supernatants were brought to pH 9-10 using 25%NH_4OH and applied to Extreut (Merck) columns. The alkaloids were eluted by CH_2Cl_2 (6mL/1g Extrelut) and the extracts were evaporated to dryness. Thus obtained residues were resolved in CH_3OH for further analysis.

Gas Chromatography/Mass Spectrometry (GC/MS): GC/MS measurements were performed after Berkov and Philipov[5]. The identities of the alkaloids were conformed by comparing the measured data with data obtained from the literature. In some cases, when no identical spectra were found, the structural type of the corresponding component was suggested only on the basis of its mass spectral fragmentation and retention data[17].

Quantification of Alkaloids: For quantification, an FID-detector was used after calibration with known amounts of hyoscyamine and scopolamine standards. GC was performed on a Hewlett Packard 5890 equipped with a HP-1 column (30m x 0.25 μm). The flow rate of the carrier gas (N_2) was 0.8mL/min^{-1} and the splitting ratio was 1:100. The temperature program was 150-270° C at 6°C/min^{-1} and held at the final temperature for 15 min. The flame ionization detector was used at 300°C and the injector temperature was 280°C.

RESULTS AND DISCUSSIONS

The Influence of Pvy Infection on the Growth of *D.metal* Plants: The data in table (1) and figure (1) illustrate the morphological difference between healthy and PVY infected *D. metal*. It was found that the highest rate of branching was obtained in infected plant being 6 branches $plant^{-1}$, while healthy plants gave 4 branches $plant^{-1}$. The number of leaves per plant was influenced by PVY infection; where 50 leaves $plant^{-1}$ were obtained in infected plant while in the case of healthy ones it reached 65 leaves $plant^{-1}$ after 6 months of cultivation. With regard to the effect of PVY, data recorded in table 1 show a decrease in plant height; 42 cm was obtained in comparison with healthy plant at 59.0 cm. Inoculation with PVY in *D.metal* resulted in a decrease of 31% in leaf area. The fresh and dry weight of *D.metal* plant was negatively influenced by PVY infection. The weight of fresh and dry shoots was decreased by 38 and 54% respectively.

All the various morphological and biochemical characteristics of PVY infection are induced indirectly by the virus. Many workers described differences in composition or in rate of some process between healthy

Table 1: Effect of PVY infection on morphological characteristics of *D.metal.*

Treatment	Rate of branching $plant^{-1}$	Number of leaves $plant^{-1}$	Leaf area (cm)	Plant height (cm)	Fresh weight (g)	Dry weight (g)
Healthy plants	2	26	3.76	59.0	55.5	9.8
PVY infected plants	3	23	2.6	42.0	34.5	4.5

Table 2: Hyoscyamine and salycilic (SA) acid contents of *D.metal* shoots infected with PVY.

Treatment	Alkaloids *Active constituent % of dry weight	Endogenous SA μg/g fresh weight
Healthy plants	0.868	25.75
PVY infected plants	0.568	120.50

*The active qonstituent was expressed as gram hyoscyamine per 100 gram dry matter (%). Alkaloids calculated as hyoscyamine.

Fig. 1: Morphological characteristics of both healthy and PVY infected *D. metal*.

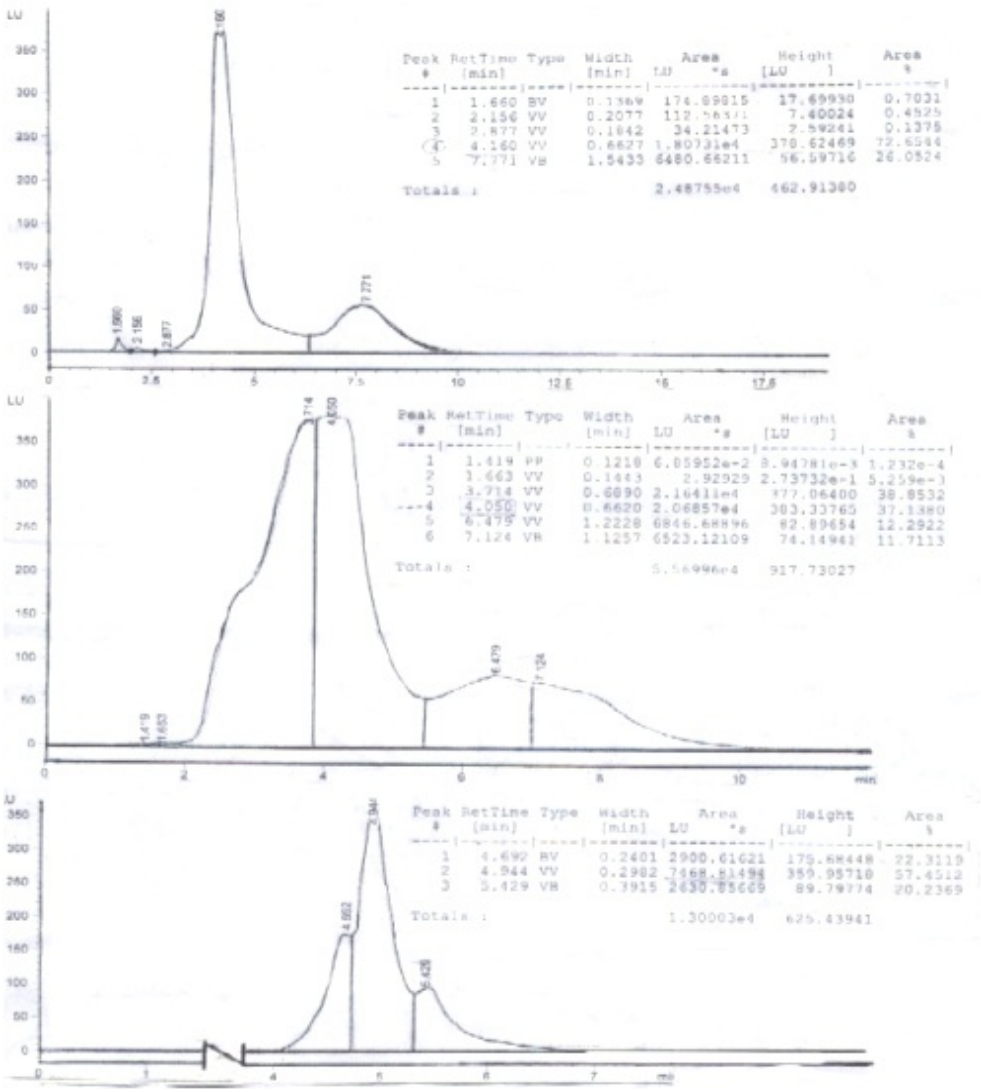

Fig. 2: HPLC quantification of endogenous SA in induced *D.metal* plants infected with PVY.
* Retention time (Ret time) of standard SA- 5± min
* Arrow refer to data of peak ranged in retention time range
A) Standard SA B) Healthy plant C) Infected plant.

Table 3: Hyoscyamine content in healthy and PVY infected *D.metal.*

Treatment	Alkaloids*		Endogenous SA** µg/g fresh weight	
D.metal origin	Healthy plants	PVY infected plants	Healthy plants	PVY infected plants
Leaves	0.95	0.72	25.75	120.50
Stem	0.75	0.60	---	---
Flowers	0.70	0.50	---	---
Seeds	0.42	0.21	---	---
Roots	0.10	0.07	---	---

*The active constituent was expressed as gram hyoscyamine per 100 gram dry matter (%). Alkaloids calculated as hyoscyamine.
** Endogenous SA induced by virus infection.

and virus infected tissues. Effects commonly found were: (i) a decrease in rate of photosynthesis often associated with a decrease in photosynthetic pigments, chloroplasts, ribosomes and fraction I protein; (ii) an increase in respiratory rate; (iii) an increase in the activity of certain enzymes, particularly polyoxidase and the accumulation of oxidized polyphenols and (iv) a decreased or increased activity of plant growth regulators. Many of the changes in host plant metabolism noted above are probably secondary consequences of virus infection, not essential for virus replication. In many virus diseases, the general pattern of metabolic change appears to resemble an accelerated aging process. There are many variables to be taken into account when using intact plant organs. The following discussion is concerned mainly with changes taking place in leaves, because these constitute most of the herbaceous host plant. More viruses are usually produced in them and they are most often used for experimental work, i.e. A mosaic virus infection of *Solanum khasianum Clarke*, reduced the fruit content of solasonine (a medically useful alkaloid) to about one-half[20].

Viruses are economically important only when they cause some significant deviation from normal growth of the plant, reduction in plant size induced by virus infection, plant height, size of leaves, flowers and roots and a shortening of petioles and internodes. A reduction in total medically useful alkaloids of leaves is a common feature and an important economic aspect of virus disease. The decrease in alkaloid contents may be due to reduction in plant total fresh weight specially leaves.

Quantification of Endogenous Salycilic Acid (SA): Figure 2 refers to the peaks obtained using HPLC, desired peak was obtained at a retention time similar to that of the standard. These peaks were used to calculate endogenous SA based on the area under peak. The results obtained from quantification of endogenous SA in infected *D.metal* gave highest level 2.15 µg/g of fresh weight compared with healthy ones which gave 1.5 µg/g of fresh weight.

In the present study, a GC/MS procedure was applied for the identification of alkaloids in the healthy and infected *D.metal* plants. Quantitative investigation of hyoscyamine revealed that *D.metal* accumulated relatively high hyoscyamine as compared to other Datura species. This result is in agreement with a previous report by Beresford and Woolley,[3]. The PVY infection caused a noticeable decrease in the hyoscyamine content of the different organs of both healthy and infected plants (Table 3). As shown in the table, the highest hyoscyamine content was found in healthy leaves (0.95 mg/100g DW) while the lowest content was found in the infected roots (0.07 mg/100g DW).

Data in table 4 show that more than 15 and 12 compounds in the alkaloid fractions of the healthy and infected plants were identified respectively. It showed the characteristic mass spectral fragmentation of the tropane alkaloids and their metabolites. Up to now, tropine (± hyoscyamine), hyoscyamine and scopolamine have been reported for the species[3, 14, 11]. To our knowledge, 5 infected shoots were identified. The characteristic alkaloids of *D.metal* are tropanol esters of a range of acids. Alkaloid 3-tigloyloxy-6-propianyloxy-7-hydroxytropane (isomeric tropine[24]) appeared as double peaks in GC/MS with identical mass spectra. This was suggested on the basis of their retention data reported in the homologous tropine esters occur in considerably higher amount as the 3- isomers[24, 22].

Tropine, psuedotopine, methyleogonine, littorine and 6-hydroxy hyoscyamine, alkaloids for solanaceae were identified in the infected shoots of *D.metal*. These alkaloids have not been detected in the healthy shoots. Previously they were characterized in inducing transformed infected shoots of *D.metal*. The structure of these compounds is conformed by a comparison of its mass spectrum and those of the reference compound from database NIST.98. These alkaloids were characterized in genetically transformed root cultures of *D. stramonium*[4]. Erythraxylaceae[11]. The rest of alkaloids have been identified according to their fragmentation pattern reported in the literature as indicated in table 4. Occurrence of apo-derivatives as

Table 4: A Comparison between the alkaloid content in both healthy and infected *D.metal* by PVY presented as % of the total ion current.

Alkaloid compound	RI (min)	Healthy shoot	Infected shoot	MS reference
Tropane	2.5	---	0.12	Witte *et al*., 1987
Psuedotropine	2.6	---	0.10	Witte *et al*., 1987
Methyleogonine	4.8	---	0.10	Berkov *et al*., 2003
Cuscohygrine	6.8	0.1	---	Witte *et al*., 1987
3-Hydroxy-6-tigloyloxy tropane	9.6	2.64	1.25	Witte *et al*., 1987
3-Tigloyloxy-6-propionyloxy-7-hydroxy tropane	10.8	2.15	1.25	Berkov *et al*., 2003
Phenylacetoxy tropane	11.2	0.25	---	Ionkova *et al*., 1994
Apohyoscyamine	12.4	1.00	0.15	Witte *et al*., 1987
Aponorhyoscyamine	12.8	0.15	---	Ionkova *et al*., 1994
Aposcopolamine	14.1	0.05	---	Witte *et al*., 1987
Littorine	14.7	---	0.12	Witte *et al*., 1987
Hyoscyamine	14.7	34.3	20.2	Witte *et al*., 1987
Norhyoscyamine	14.9	0.18	---	Ionkova *et al*., 1994
3-(3-Formyloxytropoyloxy tropane)	15.3	0.1	---	Ionkova *et al*., 1994
3-(3-Acetoxytropoyloxy tropane)	16.0	1.05	0.45	Philipov and Berkov, 2002
Scopolamine	16.4	9.15	5.25	Witte *et al*., 1987
3,6-Ditigloyloxy-7-hydroxytropane	16.9	1.92	0.75	Witte *et al*., 1987
7-Hydroxyhyoscyamine	17.2	0.1	---	Ionkova *et al*., 1994
6- Hydroxyhyoscyamine	17.5	---	0.15	Ionkova *et al*., 1994
6-Tigloyloxyhyoscyamine	22.0	0.89	---	Witte *et al*., 1987

- The area of GC/MS peaks depends not only the concentration of the corresponding compounds but also on the intensity of their mass spectral fragmentation, so the data given in the table is not a true quantification but can be used for comparison between samples which is the objective of this work.
- EIMS 70eV, m/z (ref. int):317.

apohyoscyamine and aposcopolamine may be artifacts from the isolation and CG procedures as discussed elsewhere[24, 12].

In tobacco SA levels increase as much as 180-fold after local lesion infection[10]. and it was found that free and bound SA are produced around the infection site, where as only free SA is detected region[15]. A similar increase in SA was measured in cucumber phloem exudates following infection with Tobacco necrosis virus (TNV), necrogenic pathogen that induces systemic acquired resistance (SAR) in cucumber TNV-infection led to a dramatic rise in SA level in phloem which is preceded by the development of SAR[16]. The concentration of SA in the phloem sap in cucumber plants before resistance ranged between 0.2 and 7 µm in infected plants depending on the pathogen compared to between 0 and 0.7 µm in noninfected plants[16]. As well as the host plants in pepper plants infected with PVY it was observed that the level of endogenous SA has been in low level in comparison with cucumber plants infected with *Zucchini yellow mosaic potyvirus* (ZYMV)[2]. This finding suggests that SA could act as a secondary messenger analogue capable of activating the SAR signal transduction pathway independent of SA accumulation[13].

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