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DISTRIBUTION OF ATROPINE AND SCOPOLAMINE IN DIFFERENT ORGANS AND STAGES OF DEVELOPMENT IN *DATURA STRAMONIUM* L. (SOLANACEAE). STRUCTURE AND ULTRASTRUCTURE OF BIOSYNTHESIZING CELLS

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This paper reports a study of the alkaloid content of different parts of *Datura stramonium* L. (Solanaceae) in various stages of its growth and development, and the location, structure and ultrastructure of alkaloid-biosynthesizing cells (idioblasts) in different parts of naturally grown and cultured plant material. TLC, HPLC, and GC/MS methods were used for alkaloid assays. The results showed that alkaloid production starts from the end of the second week after seed germination, increases in different organs up to the tenth week of growth, and then decreases. Leaves and capsules showed the highest alkaloid content in the vegetative and generative stages, respectively. In leaves the alkaloids decreased rapidly in the generative stage. The highest alkaloid content was recorded in vegetative leaves, followed in descending order by vegetative petioles, generative and vegetative stems, generative petioles, generative roots, generative leaves, vegetative roots and mature seeds. The organs as well as calli derived from different leaf parts were examined for the presence of idioblasts by microscopic and cytochemical methods. Idioblasts were present only in semi-hyaline callus originated from leaf base; they were spherical or oval, with a thick cell wall and large central vacuole. These observations should prove helpful in attempts to produce specific alkaloids in naturally grown plants and cell cultures.

Key words: *Datura stramonium*, tropane alkaloids, idioblast, atropine, scopolamine.

INTRODUCTION

Many studies show that certain genera of Solanaceae plants, particularly *Datura stramonium* L., produce a range of biologically active alkaloids, including tropane alkaloids (for review see: Sato et al., 2001). The major alkaloids are hyoscyamine (generally the most abundant) and scopolamine; atropine may be formed from hyoscyamine by racemization during the extractive procedure. Tropane alkaloids, together with their semisynthetic derivatives, are used as parasympatholytics that competitively antagonize acetylcholine. These alkaloids have spasmolytic and anesthetic properties (Sato et al., 2001).

In recent decades, methods aimed at production of natural compounds in vitro have employed callus induction and differentiated organ culture (Parr et al., 1990). Cultures show stable production of the compounds, which typically resemble that of the parent plant, both qualitatively and quantitatively (Robins et al., 1991). However, levels of alkaloid production have been observed to differ between cultured and naturally grown plants, and between different plant organs. For the development of alkaloid production for research and commercial purposes, it is important to determine the influence of the vegetative and reproductive phases on the quantity and content of alkaloids in various parts of plants and to identify the alkaloid biosynthesis cells called idioblasts (Constabel, 1983) and their location in natural plants or callus. Here we report findings on the content, quantity and timing of production of tropane alkaloids, and the location, structure and ultrastructure of idioblasts in different parts of natural and cultured plants and calli of *D. stramonium*.

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MATERIALS AND METHODS

Seeds of *Datura stramonium* were provided from the seed bank of the Forests Research Institute, Karaj, Tehran, I.R. Iran. The seeds were treated overnight with 2 mg/l naphthalene acetic acid (NAA) hormone (Sigma) to facilitate germination, and then two seeds per pot (total 300 pots) were grown in a greenhouse (25°C, 70% HR) in May–August. Sterile plants were also grown from seeds in the same conditions and in darkness on MS medium (Murashige and Skoog, 1962) supplemented with NAA (2 mg/l). The seeds were sterilized in 90% ethanol for 2 min and then in 5% calcium hypochlorite for 15 min, followed by two washes with double-distilled water. After 17–22 days the seeds were germinated and then were kept for at least 22 weeks. To determine the time and location of alkaloid biosynthesis or accumulation, the plants were harvested from the first to 20th weeks after germination during growth, flowering and fruiting. Harvested plants were divided into roots, leaves, petioles, stems, fruits and capsules for alkaloid content assay. At least 10 different individuals of the same age class were sampled, following the growth of the plants from May to August. The material was then dried at room temperature and the samples were randomized and used for alkaloid analysis. For each sample there were at least six repetitions of the analyses.

Calli of *D. stramonium* were induced from leaves collected from natural plants. Each leaf was dissected into three parts (basal, middle, tip), and each part was cultured separately on solid MS medium containing NAA (0.5 mg/l) in a 100 ml conical flask at 27°C either in continuous darkness or under a 16 h photoperiod. Four different types of calli were produced, designated as (1) hyaline, (2) green, (3) organogenetic, and (4) semi-hyaline. Parts of fresh calli were used for alkaloid determination. The procedures for alkaloid extraction, HPLC and TLC analysis were described in Payne et al. (1987) and Kitamura et al. (1992). TLC was used for initial screening of the presence of alkaloid in the samples. Then a full qualitative analysis of tropane alkaloids in the samples was performed by gas chromatography-mass spectrometry (GC/MS) to compare the sensitivity and accuracy of the method with HPLC. GC/MS analysis was essentially as described by Hartmann et al. (1986) and Witte et al. (1987). The alkaloids were identified by comparing their retention times and mass spectra with literature data (Evans and Major, 1986; Ionkova et al., 1994).

Light microscopy plus transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were used to determine the presence and properties of tropane alkaloid biosynthesizing cells (idioblasts) in calli derived from leaves. The TEM sample preparation protocol followed the methods outlined by Hayat (2000) for fixation, dehydration, resin penetration, embedding, semithin layer and thin layer preparation, and staining. The steps for preparation of SEM samples – dehydration by freeze-drying followed by carbon-coating – followed the procedure described by Audrey (1990). Cytochemical studies employed Dragendorff's and Logol's reagents for alkaloids.

TABLE 1. Percentage of atropine and scopolamine alkaloids in different parts of *Datura stramonium* plant quantified by GC/MS method

Developmental stage and organ	Atropine	Scopolamine	Total
Leaf in vegetative phase	0.037	0.090	0.127
Petiole in vegetative phase	0.080	0.042	0.122
Capsule	0.064	0.034	0.098
Stem in generative phase	0.070	0.023	0.093
Stem in vegetative phase	0.070	0.023	0.093
Petiol in generative phase	0.062	0.020	0.082
Root in generative phase	0.056	0.013	0.069
Leaf in generative phase	0.030	0.020	0.050
Root in vegetative phase	0.045	0.000	0.045
Seed	0.000	0.020	0.020

RESULTS

TIME SCALE OF ALKALOID PRODUCTION

Production of tropane alkaloids in *D. stramonium* plants was found to start from the end of the second week after seed germination. The rates of atropine and scopolamine production were similar (0.05%) at this stage. The quantity of alkaloids reached maximum at the end of the tenth week after seed germination, then gradually decreased as the plants entered the generative phase.

Variation between organs

Alkaloid content depended on the plant part and the stage of plant growth. Leaves and capsules showed the highest alkaloid content in the vegetative and generative phases, respectively. Generally the younger parts of plants contained more alkaloids than older ones. Alkaloid content decreased rapidly in leaves in

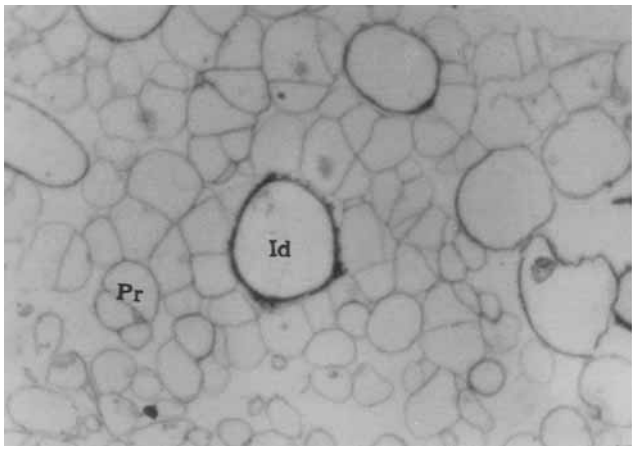

Fig. 1. Idioblast cells (Id) originated from semi-hyaline callus of *Datura stramonium*. Note their spherical or oval shape, thick cell wall and large central vacuole. Adjacent cells are smaller, condensed and smooth, whereas idioblast cells are rough. Pr – parenchyma. × 400.

the generative phase. The highest alkaloid content was determined in vegetative leaves, followed in descending order by vegetative petioles, generative and vegetative stems, generative petioles, generative roots, generative leaves, vegetative roots and mature seeds (Tab. 1).

The proportions of atropine and scopolamine alkaloids differed considerably between plant parts and developmental stages (Tab. 1). Atropine was prevalent in the vegetative period, and was much higher than scopolamine in roots, stems and petioles; in leaves, exceptionally, scopolamine was much higher than atropine.

Scopolamine was lowest (0.013%) in roots in the vegetative period, and then totally disappeared in the generative period. Atropine occurred in roots in both the vegetative (0.045%) and generative (0.056%) periods, slightly increasing in the latter. Stems were rich in atropine (0.070%) but poor in scopolamine (0.023%) in both stages. In stems, atropine was three times higher than scopolamine. Similarly, in petioles the atropine/scopolamine ratio was 2:1 (0.080:0.042%) in the vegetative stage, with the quantities falling but the ratio rising (3:1, 0.062:0.020%) in the generative stage. Scopolamine increased in leaves as they grew until the generative period, then decreased. At the beginning of flowering, leaves had the highest scopolamine content (0.090%), after which it decreased rapidly to 0.020%. Atropine occurred in leaves in both periods, but its quantity (0.030–0.037%) was much lower than scopolamine. In seeds there were small amounts of scopolamine (0.020%) but no trace of atropine. Capsules had 0.064% atropine and 0.034% scopolamine alkaloids.

Structure and ultrastructure of idioblasts

Different organs of plants in the vegetative phase as well as the calli derived from different parts of leaf were investigated to detect cells responsible for alkaloid biosynthesis and accumulation (idioblasts). Mersey and Culter (1986) used different methods, particularly TEM and SEM, to show idioblast cells very clearly in *Catharanthus roseus*. The idioblast cells in *Datura stramonium* appeared spherical or oval, with a thick cell wall and a large central vacuole. The adjacent cells to them were smaller, condensed, and had a smooth surface, whereas the idioblast cells were rough (Fig. 1).

Alkaloid assays using Dragendorff's and Logol's reagents revealed no evidence of idioblast cells in

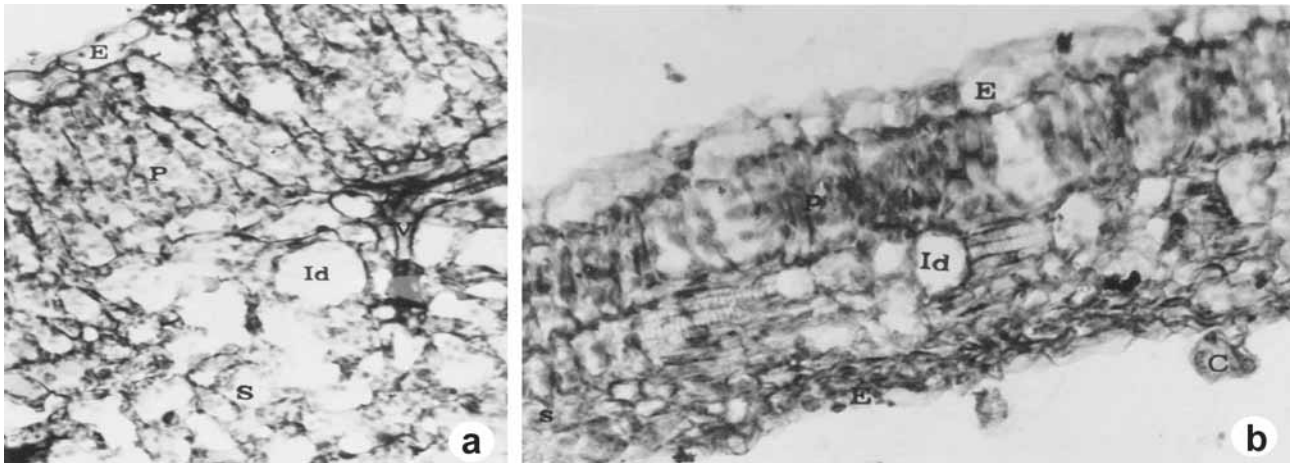

Fig. 2. Idioblast cells (Id) in leaves of *Datura stramonium* adjacent to vessel bundles and subsidiary veins, and in interstices of scalenous chlorenchyma. (**a**) Transverse section of leaf at time of inoculation, (**b**) Transverse section of leaf cultured in vitro. E – epidermis; S – scalenous chlorenchyma; C – cork. × 400.

TABLE 2. Properties of four types of callus originated from leaf explants of *Datura stramonium*

Type of callus	Origin	Color	Morphology	Shape	Average size (µm)		Specific property	Position of differentiation
					Cell	Nucleus		
Hyaline	middle part of leaf	white	rugged rigid and dense surfaces	spherical or ellipsoid	257.5 ± 45.5	190.15 ± 0.74	thin cell wall; development of vacuolar system; reach in starch	leucoplast
Semi hyaline	basal part of leaf	brownish yellow	rugged loose and fragile surfaces	various cell shapes	146.25 ± 16.52	15.63 ± 3.6	thick cell wall; development of vacuolar system; margination of organelles; low starch; enlargement of cells	idioblasts
Green	middle part of leaf (under light)	green	rugged rigid and dense surfaces	spherical or flat	140.25 ± 21.21	24.43 ± 1.57	thin cell wall; development of vacuolar system; enlargement of nucleus	chloroplasts, leucoplast and vessel element
Organo-genetic	apical part of leaf	brown	rugged rigid and dry-appearing surfaces	spherical or ellipsoid	76.08 ± 26.25	8.13 ± 0.5	thin cell wall; development of vascular system; abundance of calcium oxalate crystals; formation of root primordias	xylem with production of root organ

roots. In stems, some characteristic idioblast cells were seen under the epidermis. Leaf histology showed some idioblast cells adjacent to the vessel bundles and subsidiary veins, and in interstices of scalenous chlorenchyma. In the petiole, idioblast cells occurred in the collenchyma tissue (Fig. 2).

Four different calli, distinguished as (1) hyaline, (2) green, (3) organogenetic and (4) semi-hyaline, were analyzed for idioblast cells. Table 2 shows the properties of the four types of calli produced from leaf explants of *D. stramonium*. Medial parts and tips induced hyaline, green or organogenetic callus in darkness or under a 16 h photoperiod; none of them contained idioblast cells. Under a 16 h photoperiod, basal parts induced slower-growing, semi-hyaline callus. Anatomical and cytochemical analysis revealed that only this semi-hyaline callus contained idioblast cells, producing alkaloids with almost similar amounts of atropine (0.0087% v/v) and scopolamine (0.0095%) (Fig. 3).

DISCUSSION

We demonstrated that tropane alkaloid production starts from the second week after seed germination, peaks at the tenth week, and then declines gradually. This is in accordance with Klan's (1931) finding that the alkaloid content of *Hyoscyamus niger* plants increases during the vegetative period and then decreases in the generative period, and with the suggestion that at fruiting time the alkaloids are decomposed and recycled to form important substances in, for example, seeds.

The level of tropane alkaloids in roots was lower than in aerial organs, as also reported by Miraldi et al. (2001). Atropine is the predominant alkaloid in *D. stramonium* in the generative period (Papadoyannis, 1995; Oshima et al., 1989). In contrast, scopolamine was predominant during all development stages of *D. metel* plants (Afsharypuor et al., 1995). In our study, atropine predominated over scopolamine in all organs that passed the vegetative stage, except for seeds. Scopolamine increased with the increase in atropine content. Scopolamine was not found in roots in the vegetative period, and in the generative period only a tiny amount was detected. In the literature there is only one report concerning alkaloid content in stems of *D. stramonium* (Miraldi et al., 2001). We found considerable quantities of atropine and scopolamine in stems of *D. stramonium* in both stages. In leaves in the vegetative stage we measured scopolamine levels much higher than those of atropine, in agreement with many authors (Duez et al., 1985; Oshima et al., 1989; Papadoyannis 1995) but in contradiction to Miraldi et al. (2001). In stems in the generative stage there was slightly less scopolamine than atropine. Atropine was completely absent from seeds, and only very low scopolamine content was detected in

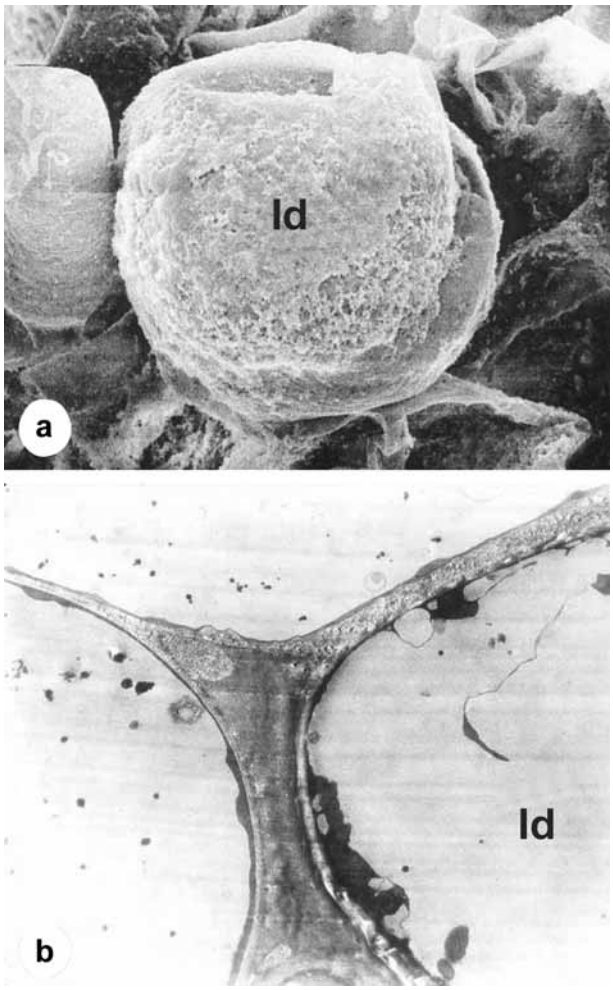

Fig. 3. Micrograph of idioblast cells in semi-hyaline callus originated from basal part of leaf of *Datura stramonium*. (**a**) SEM, × 1400, (**b**) TEM, × 4400.

them. This is in contrast to previous reports (Oshima et al., 1989; Duez et al., 1985; Papadoyannis, 1995) that seeds are one of the plant parts with the highest content of tropane alkaloids.

Our observations of different organs and calli derived from leaves clearly indicate several distinct characteristics of idioblasts. The most useful diagnostic characters are the large vacuole and its contents, which are highly refractile and not easily miscible with the surrounding medium. Mersey and Culter (1986) have shown that idioblast size varies, however, and that some mesophyll protoplasts contain small spherical droplets of refractile, yellow fluorescent material within the vacuole. It is not clear if these cells represent an intermediate stage in the differentiation of idioblasts or whether all mesophyll cells accumulate these materials under certain circumstances. In this study we found that calli containing idioblasts grew more slowly than other calli. This has also been shown in other studies (Mersey and Culter, 1986; Lindsey and Yeoman, 1983), which have suggested that there probably is a general relationship between growth rate, differentiation, and secondary product accumulation.

This study confirmed that alkaloid production starts from the end of the second week after germination, peaks at the tenth week, and decreases gradually during the generative stage. The work also determined the proportion of alkaloids in each part or organ. These data can help growers focus on particular organs and regulate harvesting time to obtain maximum alkaloid content. We also found idioblast cells in semi-hyaline calli derived from basal parts of leaf cultured under a 16 h photoperiod; thus it is possible to obtain tropane alkaloids from cell cultures under appropriate conditions.

We note the importance of appropriate conditions such as medium composition, plant genus or lines, and genetic engineering to increase the accumulation of scopolamine as demonstrated by Lanoue et al. (2004) and Dechaux and Boitel-Conti (2005).

The temporal and spatial distribution of tropane alkaloid and the description of the structure and ultrastructure of alkaloid-biosynthesizing cells (idioblasts) in different parts of naturally grown and cultured plant material provide valuable data for future work on scaling-up tropane alkaloid production. These data in combination with optimized conditions and promising biotechnological processes seem to be the best way to produce specific alkaloids in naturally grown plants and cell cultures.

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