Calystegine distribution in potato (*Solanum tuberosum*) tubers and plants

Ronald Keiner, Birgit Dräger *

Institute of Pharmaceutical Biology, Martin-Luther-University Halle-Wittenberg, Hoher Weg 8, D-06120 Halle, Saale, Germany

Received 19 April 1999; received in revised form 16 July 1999; accepted 14 September 1999

**Abstract**

Potato plants contain calystegines in leaves, stems, flowers, fruits and roots. Calystegines A₃ and B₂ are the main constituents. Highest concentrations were measured in sprouts emerging from the tubers. In 3 mm long sprouts, 3.3 mg total calystegines per g fresh mass were detected. Dormant tubers directly after harvest contain less calystegines in all parts than sprouting tubers. Flowers and young leaves are the aerial plant tissues with the highest calystegine concentration, i.e. 150 mg total calystegines per g fresh mass. Calystegine levels did not rise when sprouts were wounded. Tropinone application to sprouts and aerial tissues lead to an accumulation of pseudotropine and not to tropine. That indicates that stereospecific tropinone reduction is active in potato.

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**Keywords**: Potato; *Solanum tuberosum*; Calystegine accumulation; Tropane alkaloid biosynthesis; Tropinone reductase

1. Introduction

Calystegines, a class of nortropane alkaloids, have been described in potato (*Solanum tuberosum* L.) leaves and tuber skins [1]. However, higher concentrations were measured in sprouting potatoes [2].

Calystegines possess considerable activities as glycosidase inhibitors [3]. The inhibitory activity depends on the number and position of hydroxyl groups on the nortropane skeleton and varies with specific glycosidases. Up to now 14 different calystegine structures have been published (Fig. 1), among them N-methyl derivatives, which have tropane skeletons. Most of the calystegines were isolated from Solanaceae [3].

We became interested in calystegines because of our research on tropane alkaloid biosynthesis. It is assumed that calystegines originate from the tropane alkaloid pathway [4] (Fig. 2). In order to prove this hypothesis plant tissues were investigated for high biosynthetic activity. Potato tissues of several developmental stages were analysed.

Here are presented detailed calystegine measurements in potatoes of the cultivar Liu and first attempts to investigate the biosynthetic pathway by feeding the metabolite tropinone.

2. Material and methods

2.1. Plant and tissue cultivation

Potatoes, cultivars Adretta and Arkula (early tuberisation) and cultivar Liu (later tuberisation), were purchased from a local provider in early September, and it was ensured that they were freshly harvested and not treated with sprouting inhibitors. They were kept dark at 4°C. Under these conditions they did not sprout within 8 months. For sprouting induction tubers were transferred to room temperature and kept dark.
After the onset of sprouting, samples were taken every second day.

Potato tubers cut longitudinally show a ring of vascular tissue 0.5–1.5 cm below the skin. The storage tissue inside this ring, termed ‘pith’, was examined. When the outside cork layer of the tubers was cut off by a scalpel < 1 mm in thickness, it was termed ‘skin’ and when it was cut off with a scalpel 2 mm in thickness we called it ‘peel’.

Potatoes, either sprouting with up to 3 mm long sprouts or directly from the cold room were planted in April into the soil of the medicinal plant garden at the institute in Halle. Samples were taken every fifth day until aerial leaves appeared. Thereafter plants of three different sizes were analysed.

Some of the sprouting potatoes were planted in pots and kept at room temperature. They grew faster with longer internodia than outside. The stems were used for feeding experiments with tropinone.

Tobacco leaves (Nicotiana tabacum) were obtained from flowering green house plants of the medicinal plant garden.

2.2. Feeding experiments with tropinone

Tropinone (Aldrich, Deisenhofen, Germany) was 99% pure according to the manufacturer’s declaration. The purity was confirmed by GC-analysis. Further, tropine or pseudotropine were not detected. More than 0.5% tropine or pseudotropine would have been clearly visible by GC. Ten potato sprouts of length = 3 cm were cut off the tuber. The endings were dipped (5 mm immersion depth) into a solution of 5 mM tropinone, buffered with HCl to pH 7. After 24 h the endings were thoroughly washed with water to remove tropinone solution at the outside and extracted.

The experiment was repeated three times. Five whole leaves of each, potato and tobacco were treated with the same tropinone solution (3 cm immersion depth). Twelve aerial stems of plants with leaves (≈ 15 cm in length) were dipped into 5 mM buffered tropinone solution (3 cm immersion depth), and after 3, 8 and 18 h they were cut into

Fig. 1. Calystegines in S. tuberosum.

Fig. 2. Tropinone reduction as branch point in the tropane alkaloid biosynthesis. TR = tropinone reductase, PMT = putrescine methyl transferase.
four sections of equal length. The sections were extracted separately. Control sprouts, leaves and stems were kept in 5 mM KCl solution.

2.3. Extraction and analysis of calystegines

Collected tissues were weighed and lyophilised. Dry mass was determined and tissues were extracted as described [5]. In a variation of this method, strong acidic cation exchange resin I LAB (Merck, Darmstadt, Germany) was used instead of Dowex 50 WX8. Each analysis used 1–5 g fresh mass.

Isolated calystegines A3, A5, B1, B2, B3, B4 and C1, N-methyl-calystegine B2 (gifts from Dr N. Asano, Kanasawa) were available as reference compounds for GC. Instead of deoxynojirimycin [5], isolated calystegine B2 was taken for calibration of the GC method and azobenzene (100 μg/ml) was used as an internal standard. Calystegines were derivatized with silylating agents TMCS/HMDS as described before [5].

The detection limit for calystegines in extracts was \( \approx 1 \mu g/ml \) sample and reliable quantification was possible with \( > 10 \mu g/ml \) sample. With the sample preparation procedure described above the limit of quantification results as 2–5 μg calystegines/g fresh mass of tissue depending on the fresh mass used per sample.

Identity of calystegines in the potato extracts was further confirmed by GC-MS analysis in comparison to reference compounds. The TMS-derivatives of the calystegines give typical fragmentation patterns [3,5].

2.4. Extraction and analysis of tropane alkaloids

After feeding tropinone tissues were homogenised in H2O and the filtrate was evaporated to <1 ml. Fifty microlitres of ammonia were added and the volume was adjusted to 1.0 ml with water. The extracts were loaded onto columns with 1.0 g Extrelut (Merck, Darmstadt). After 15 min the columns were eluted with \( 2 \times 3 \) ml CHCl3 and \( 1 \times 3 \) ml CHCl3/MeOH 85:15. The eluate was concentrated to <1.0 ml, diluted to 1.0 ml with ethyl acetate/MeOH 1:1 and used for GC and TLC analysis.

GC for tropinone, tropine and pseudotropine was performed as described previously [6], with some alterations: column DB5, 30 m, 0.32 mm ID, 0.25 μm phase, 2 ml/min He as carrier gas. Temperature programme: start 60°C, 5°/min to 100°, 15°/min to 240°. Retention times were 8.05 min for tropinone, 8.35 min for tropine, 8.65 min for pseudotropine.

The detection limit was 10 μg/ml GC-probe for tropinone and tropine and 50 μg/ml for pseudotropine.

TLC for tropinone, tropine and pseudotropine was carried out on silica gel plates (0.2 mm layer, 60 μm particle size, Merck, Darmstadt, Germany), solvent system: CHCl3/MeOH/NH3 44:9:1, with double development. For detection the Dragedorff reagent was used with the variation according to Munier [7] using tartaric acid instead of acetic acid. The sensitivity for tropine and pseudotropine was 0.1 μg, visible as purple zone on a light yellow background.

2.5. Statistical treatment of data

In order to quantify calystegines, between three and six independent samples of each tissue were extracted. S.D. are always given in bars, in Fig. 4 they are sometimes so low that they are hardly visible. In exceptional cases (sprouts in soil, Fig. 3), when only two tissue samples were available, no S.D. is given.

The extract of each sample was analysed by GC usually twice, with 1 μl and 2 μl injection volume to account for the different concentrations of calystegines.

Data were examined for outliers. An outlier was defined as a single value that deviated more than 30% from the average calculated over all values. The limit of 30% was set after calculating several suspected outliers by the method of Nalimov. Single data with about 30% difference from the arithmetic average turned out to be outliers. These values were eliminated before calculation of S.D.

3. Results and discussion

3.1. Calystegine distribution in tubers and sprouts

Tubers and sprouts of the potato cultivars Adretta and Arkula (early tuberisation) and Liu (later tuberisation) were compared, and they did not show considerable differences in calystegine
Fig. 3. Calystegine levels in tubers and sprouts.

content in corresponding developmental stages. To reduce the number of samples, only cultivar Liu was examined in more detail.

After harvesting the tubers were completely dormant [8]. They did not sprout even if they were kept at room temperature for several weeks. The dormant tubers were examined for calystegines (Fig. 3). For subsequent experiments, tubers stored at 4°C were examined monthly. After 3 months of storage single sprouts appeared when tubers were transferred to room temperature for 2 weeks. After 5 months of storage at 4°C, transfer to room temperature resulted in intensive sprouting after 1 week. These tissues were examined (Fig. 3).

Further sprouting tissues were analysed after 8 months of storage at 4°C. At that time (April) tubers were also planted in soil and the growing plants were used for further measurements.

Calystegine A₃, B₂, and B₄ were the most prominent and could be quantified in all tissues given in Fig. 3. The presence of further calystegines was noted, and calystegines A₅, B₁, and B₃ were suspected from comparison with authentic com-
pounds and comparison with extracts from other calystegine-containing plants (Calystegia sepium, Atropa belladonna) by GC and by GC-MS. Further calystegines cannot be excluded, but they are present in minor amounts only ( > 10 μg/g fresh mass). N-methyl calystegine B₂ was not detected in any potato extract.

Within the four developmental stages highest accumulation was registered in sprouting eyes after 5 months of cold storage (Fig. 3). Sprouting start was defined by the appearance of white shoot tip primordia of < 1 mm size in the brown cork.

All of the tissues measured at that time contained more calystegines than dormant tubers, e.g. the tuber skins without eyes at sprouting start contained three times more calystegines than 5 months earlier.

Eight months after harvesting the calystegine accumulation in the sprouting eyes and small sprouts was approximately half of that after 5 months. Sprout growth was faster than after 5 months of storage. As sprout growth proceeds, the calystegine level decreases on a fresh mass basis. Longer sprouts contain more water than 3-mm sprouts (Fig. 5), but this alone is not the reason for the decrease in calystegine concentration. Calystegine accumulation decreases as sprouts elongate and does not parallel growth on a dry mass basis either.

Sprouts and sprouting potato tubers have not been investigated in detail previously.

### 3.2. Distribution in plants

The sprouting potatoes in the soil were left to grow into plants which were examined at three stages of development (Fig. 4). On the whole, values in aerial parts are much lower than in sprouting tubers. Calystegine B₄ could be detected, but was too low to be quantified reliably. Only the levels of calystegines A₃ and B₂ are given.

The leaf primordia directly under the soil surface (Fig. 4) show a decreased calystegine level compared with the sprouts in the soil (Fig. 3).

The first aerial leaves keep the calystegine level of leaf primordia, and the roots of the small plants contain about the same concentration and pattern of calystegines (Fig. 4).

While the calystegine level in the roots remains rather constant in 20-cm plants, the leaves gradually lose calystegines. Younger leaves contain more calystegines than older leaves, that has been shown for leaves of other Solanaceae as well [2].

Plants of 20 cm begin to form tubers, which contain the highest concentrations of calystegines at this stage of development.

Plants of 30–40 cm (Fig. 4) were flowering and lower leaves had begun to wilt. The tubers had almost reached their final size at that stage. Flowers extracted as a whole including petals had the highest calystegine levels recorded in aerial parts of the plants.

Besides the values given in Fig. 4 more tissues of older plants were examined, e.g. yellow leaves, wilted leaves, yellow stems, whole green fruits. In these organs calystegine levels were too low to be measured, i.e. < 5 μg/g fresh mass.

All calystegine concentrations are given on a fresh mass basis, but dry mass was determined for each sample. Typical values for each type of tissue are given in Fig. 5. Other dry mass per fresh mass relations were similar for the respective tissues. The dry mass proportion in aerial parts of the plant is lower than in tubers, but this alone does not account for the lower calystegine content per fresh mass. On a dry mass basis calystegine content in aerial parts is lower as well.

Potatoes are known to contain steroidal glycoalkaloids, e.g. solanin and chaconin, their accumulation pattern has been intensively investigated [11].
Calystegine concentrations are partially parallel to those of the glycoalkaloids. Tuber tissues known for high accumulation of glycoalkaloids are skins (up to 640 µg/g fm), eyes and sprouts (≈ 5 mg/g fm), the glycoalkaloid concentrations being highly dependent on the cultivar. Tuber pith contains only low amounts of glycoalkaloids (up to 60 µg/g fm), the same is true for calystegines. In aerial parts of the potato plants again the flowers contain the highest concentrations of glycoalkaloids (up to 5 mg/g fm), but on the whole, aerial parts, leaves in particular, contain more glycoalkaloids than tubers while calystegine concentrations are highest in particular tuber tissues. Another difference is that glycoalkaloids accumulate in tuber skins when tubers are illuminated [11]. Calystegines in skins do not rise with illumination, and when tubers are left to sprout under illuminations the sprouts are green, stunted and contain less calystegines that the equivalent sprouts in the dark (data not shown).

3.3. Distribution within the sprouts

Because upper leaves always contain more calystegines than leaves from lower parts of the plants, sprouts were dissected and analysed (Fig. 6).

Calystegines accumulate predominantly in the tip region. The lowest parts of the sprouts contain

*Fig. 4. Calystegine levels in roots and aerial parts of *S. tuberosum*.
higher calystegine levels probably due to higher concentration in root primordia. Higher calystegines concentrations in tips and root tips were repeatedly measured with sprouts > 100 mm, but in sprouts of 20 mm no difference could be found between tips and middle parts.

Calystegines seem to concentrate in young meristematic tissues. This leads to the question of the biological significance of calystegine accumulation. They could be protecting compounds as discussed for other glycosidase inhibitors [12], and we wondered whether they are inducible like phytoalexins. We tested the response to mechanical stress by wounding sprouts with a scalpel. The surface of 3 cm long sprouts, 4 mm in diameter was scratched vertically to 1–2 mm depth. The sprouts were analysed every day up to 6 days after wounding and showed no alteration of calystegine content. Six days after wounding the scratch had formed a brown cork and side sprouts began to proliferate. Thus wounding had an effect on plant growth but not on calystegine accumulation.

Under these conditions potato phytoalexins have been described to accumulate within 24 h [13]. Typical phytoalexins in response to tuber wounding are the sesquiterpenes rhishitin, lubimin, and solavetivone, the compounds are hardly detectable in healthy tissues. Wounding also leads to a higher accumulation of glycoalkaloids [11]. In our experiment calystegines are no phytoalexins in the sense that they appear or rise in concentration after biotic or abiotic stress. If calystegines serve as defence compounds they are constitutive protectants for young tissues. Experiments with insects to investigate feed deterrence are on the way.

3.4. Tropinone as metabolite

As sprouts were the tissues with the highest calystegine accumulation (Fig. 3), they were examined for the first putative step of calystegine biosynthesis, the reduction of tropinone (Fig. 2).

In a first set of experiments tropinone was fed to potato sprouts. Potato leaves were also examined and tobacco leaves were taken as a control. After 24 h of tropinone application to potato sprouts and leaves, pseudotropine was detected besides
non-metabolised tropinone by TLC and GC. Tropine could not be detected. In control tissues none of the metabolites could be found. Tobacco leaves fed with tropinone solution showed tropinone in GC and TLC, but neither pseudotropine nor tropine. Here nicotine was visible on TLC (results not shown).

After 3 h of tropinone application to potato stems, tropinone was detected in the lowest section (≈ 4 cm) only. After 8 h tropinone was found in the lowest and in the next higher part, but no pseudotropine or tropine could be found. After 18 h tropinone was detectable in all sections, and in the lowest section pseudotropine, but no tropine, was identified. In our view the pseudotropine concentration is the result of the formation minus the further metabolism to calystegines, so that pseudotropine accumulates, because with the high substrate supply the tropinone reduction is very active.

In summary there is transport of tropinone in tobacco and potato tissues, but tropinone is only reduced to pseudotropine in potato sprouts, stems and leaves. The reduction is stereospecific, with only pseudotropine, not tropine accumulating. This is in accordance with the finding of two separate tropinone reductases in other tropane alkaloid containing Solanaceae [6,14]. The tropine forming enzyme (TR I, Fig. 2) leads to hyoscyamine and scopolamine, which have never been found in S. tuberosum. It is suspected that the pseudotropine forming enzyme (TR II) takes part in calystegine biosynthesis.

These experiments are taken as first evidence that tropinone and pseudotropine are metabolites for the biosynthesis of calystegines in potato. The next aim is to elucidate the subsequent steps and to prove the metabolic pathway by characterisation of the enzymes involved.

Acknowledgements

We wish to thank Dr Naoki Asano, Kanasawa, Japan, for the gift of isolated calystegines. Technical assistance by Ursula Ködel is gratefully acknowledged. R.K. is grateful for a grant from the Landesgraduiertenförderung Sachsen-Anhalt.

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