Accumulation of Tetracoumaroyl Spermine in *Matricaria chamomilla* during Floral Development and Nitrogen Deficiency

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The new natural polyamine conjugate 1*N*,5*N*,10*N*,14*N*-tetracoumaroyl spermine (tetracoumaroyl spermine) recently isolated from chamomile (*Matricaria chamomilla* L.) flower heads is applicable for the treatment of several human disorders such as depression and anxiety. High variability in the level of tetracoumaroyl spermine is found in commercial tisanes. Accumulation of tetracoumaroyl spermine was tested during floral development, and nitrogen deficiency was chosen as its putative limiting environmental factor. It was observed that tetracoumaroyl spermine is present mainly in tubular flowers, reaching its maximal content during the 3rd phase of flowering when the corollae of tubular flowers start to open. The later observed decrease could result from a release of pollen that also contains a considerable amount of tetracoumaroyl spermine. It is likely that tetracoumaroyl spermine plays an important role in pollen development, and so, despite overall N-deficiency in the plants, tetracoumaroyl spermine is accumulated at the same or even higher rate than in the flowers of the N-sufficient control.

Key words: Matricaria chamomilla, Tetracoumaroyl Spermine, Nitrogen Deficiency

Introduction

The anthodia of chamomile (*Matricaria chamomilla* L.) are widely used as a popular ingredient for herbal teas. The chamomile drug produced from floral heads is traditionally used for its several medicinal properties including antiplatelet, antioxidant, anti-inflammatory, antimutagenic or cholesterol-lowering activities, as well as antispasmotic and anxiolytic effects (McKay and Blumberg, 2006). However, human studies are still limited, so further research into these beneficial properties is essential. The main active constituents present in the flowers are flavonoids (apigenin and its derivatives), coumarins (herniarin and umbelliferone), and the constituents of essential oil: mainly chamazulene, farnesene, (-)-α-bisabolol, and dicycloether (Schilcher *et al.*, 2005).

Secondary metabolites such as coumarins and 1,2-benzopyrans occur in the majority of plant species and are often present in small quantities in all plant organs, but they accumulate in considerable amounts in some plant organs such as in roots, reproductive organs, and surface tissue cells (Smyth *et al.*, 2009), especially in stress conditions (Dewick, 2002). Precursors of coumarins, for example *o*-hydroxycinnamic acids (or phenolic acids), are able to conjugate with polyamine compounds such as putrescine, spermidine or spermine. The formation of the amide linkage between a polyamine and phenolic acid is controlled by a class of specific transferases. Spermine hydroxycinnamoyl transferase links the CoA-activated carboxy group of a phenolic acid with a spermine amino group (Hedberg *et al*., 1996; Martin-Tanguy, 2001). Polyamine conjugates with phenolic compounds have been found in a wide range of plant species of several families such as Fabaceae, Asteraceae, Amaryllidaceae, and Araceae (Walters, 2003; Yamamoto *et al.*, 2002; Youhnovski *et al.*, 2001). They are the predominant phenolic compounds in the reproductive organs, pollen, and seeds, but have also been identified in roots, stems, or leaves in small amounts (Edreva *et al.*, 2007). The biological properties of these compounds result from the combination of characters of their parent compounds. For example, the ability to quench reactive oxygen species (like H_2O_2, $^1O_2$) is due to scavenging properties of

both *p*-coumaric acid and polyamines (Edreva *et al.*, 2007). However, their exact *in vivo* function in plants has not been clearly established. It is supposed that they represent the source and transport forms, respectively, of the parent compounds. Other protective metabolites, phenylamides, are used by plants in the processes related to growth and development of their reproductive organs and pollen grains (Aribaud *et al.*, 1998), antifungal activities, hypersensitive response to pathogen attack (Walters *et al.*, 2001), and in reactions to abiotic stress, *e.g.* UV irradiation (Groppa and Benavides, 2008; Youhnovski *et al.*, 2001).

The polyamine conjugate 1*N*,5*N*,10*N*,14*N*-tetrakis[3-(4-hydroxyphenyl)-2-propenoyl]-1,5,10,14-tetraazatetradecane (tetracoumaroyl spermine, Fig. 1) was discovered by Yamamoto *et al.* (2002), who isolated it from chamomile tubular flowers and pollen and found it in flower extracts of six species of Asteraceae. The identified compound belongs to the group of conjugated polyamines that are non-peptide antagonists of tachykinin NK_1 receptors and have positive effects against depression and anxiety. Most of the NK_1 antagonists reported so far are synthetic products, whereas tetracoumaroyl spermine is a natural compound.

The aim of the present work was to compare tetracoumaroyl spermine levels in commercially available chamomile tea bags, to determine its distribution throughout the plant and flower heads, as well as to evaluate its accumulation during ontogenesis of the flower heads under normal nutrition conditions and during nitrogen deficiency.

Material and Methods

Plant material and growth conditions

To screen the tetracoumaroyl spermine content in chamomile tea bags available on the Slovak market, the following most common commercial products were used:

- Agrokarpaty Plavnica s. r. o., Slovakia, "Rumanček BIO", 20 bags, 1.5 g per bag;
- Baliarne obchodu Poprad a. s., Slovakia, "Rumanček pravý", 20 bags, 1.5 g per bag;
- Belin Z. P. Ch., Poland, "Rumianek", 30 bags, 1.3 g per bag;
- Fytopharma a. s., Slovakia, "Rumančekový čaj", 20 bags, 1 g per bag;
- Herbex s. r. o., Slovakia, "Rumanček pravý", 20 bags, 3 g per bag;
- Klember a spol. s. r. o., Slovakia, "Rumanček pravý", 20 bags, 1.5 g per bag;
- Leros s. r. o., Czech Republic, "Heřmánek", 20 bags, 1 g per bag.

For further experiments, two chamomile (*Matricaria chamomilla* L.) cultivars, diploid cv. 'Novbona' and tetraploid cv. 'Lutea', were grown under experimental field conditions. The flower heads were collected during five phases of development:

1. The involucral bracts are differentiated, but neither the tubular nor ligulate flowers, respectively.
2. The ligulate flowers are still coiled, but longer than the valuated top of the anthodia; the corollae are parallel with the axis of the anthodia.
3. The corollae of ligulate flowers are developed; tubular flowers are starting to bloom.
4. The ligulate flowers are still blooming; about half of the tubular flowers are opened.
5. The ligulate flowers are starting to bend towards the stem; nearly all tubular flowers are opened.

For the nitrogen deficiency experiment, the tetraploid cultivar 'Lutea' was used. The plants were grown in half-strength Hoagland's solution and kept in a growth chamber under controlled conditions [24 °C, 70% relative humidity, 16 h/8 h photoperiod at 180 μmol/(m^2 s), light colour 6500 K]. At the start of rapid stem elongation and before development of floral buds, 50% of the plants were transferred to the N-free medium [modified half-strength N-free Hoagland medium in which $Ca(NO_3)_2$ was replaced with $CaCl_2$]. The flower heads were collected at phase 3 (10–15 d of nitrogen deficiency, depending on the development of the anthodia) and phase 4 (12–17 d of nitrogen deficiency). The collected plant material was dried at laboratory temperature. Dry weight

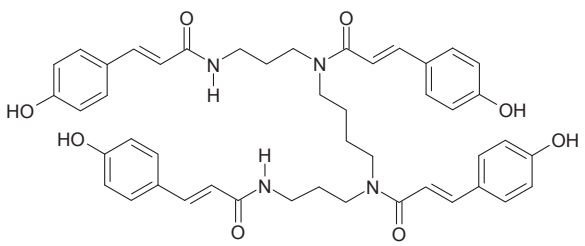

Fig. 1. Chemical structure of 1*N*,5*N*,10*N*,14*N*-tetrakis-[3-(4-hydroxyphenyl)-2-propenoyl]-1,5,10,14-tetraazatetradecane (tetracoumaroyl spermine).

(DW) was determined after drying to constant weight at 105 °C.

Determination of nitrate and total nitrogen content

Spectrophotometric determinations of the nitrate content in leaf crude extracts were performed on alternate days during the nitrogen deficiency experiment, following the method described by Cataldo *et al.* (1975) with slight modifications (Pajuelo *et al.*, 2002): 200 μL of 5% (w/v) salicylic acid dissolved in 96% (w/v) sulfuric acid were added to 50-μL aliquots of the leaf crude extracts and left to react for 20 min. Then 2 M NaOH (4.75 mL) was added to the reaction mixtures, and after cooling the absorbance was read at 405 nm. The calibration curve of known amounts of nitrate dissolved in the standard extraction buffer was used for analytical determinations. Controls were set up without salicylic acid. The total nitrogen (amino-N) content was determined by the standard Kjeldahl method (Kirk, 1950) after 12 d of nitrogen deficiency.

Amino acid analyses

The content of free amino acids in flower heads and leaf tissue was determined in the plant material ground to a fine powder, extracted with 80% methanol, dried, and re-dissolved in 1 M sodium borate buffer (pH 9.0) containing 0.02% sodium azide. Amino acids were assayed following pre-column derivatization with diethyl ethoxymethylenemalonate (DEMM) for 50 min at 50 °C (Alaiz *et al.*, 1992). Derivatization was followed by reversed-phase high-performance liquid chromatography (HPLC). The system included an Ecom LCD 3001 pump (Praha, Czech Republic), an Ecom LCD 2084 UV-VIS detector, and a 300 × 3.9 mm I.D. reversed-phase column (Nova-Pack C_{18}, 4 μm; Waters, Milford, USA). Resolution of amino acid derivates was accomplished using a binary gradient system: the two solvents were (A) 19% acetonitrile and (B) 70% acetonitrile. The solvent was delivered to the column at a flow rate of 0.7 mL/min as follows: 0–10 min, solvent A; 10–25 min, linear gradient to A/B (50:50); 25–40 min, linear gradient to A/B (25:75); 40–50 min, elution with A/B (25:75); 50–55 min, linear gradient to solvent A. Detection was at 280 nm. Levels of amino acids were determined using commercial standards (Sigma-Aldrich, St. Louis, MO, USA), and the results obtained were re-calculated to a fresh weight basis.

Analyses of tetracoumaroyl spermine

Plant material was homogenized in a mortar, and tetracoumaroyl spermine was extracted with methanol at laboratory temperature and assayed by HPLC as above with a 250 × 4 mm I.D. reversed-phase column (Separon SGX C_{18}, 7 μm; Tessek, Praha, Czech Republic). Resolution of tetracoumaroyl spermine was accomplished using a binary gradient system: the three solvents used were (A) acetonitrile/H_2O/H_3PO_4 (19:80:1), (B) 70% acetonitrile, and (C) 90% acetonitrile. The solvent was delivered to the column at a flow rate of 0.7 mL/min as follows: 0 min, A/B/C (65:35:0); 0–15 min, linear gradient to A/B/C (50:50:0); 15–20 min, linear gradient to A/B/C (0:100:0); 20–25 min, linear gradient to A/B/C (0:0:100); 25–30 min, elution with A/B/C (0:0:100); 30–35 min, linear gradient to A/B/C (65:35:0). Detection was at 300 nm. All analyses were performed with fresh extracts, because of the low stability of tetracoumaroyl spermine. Calibration was done with isolated tetracoumaroyl spermine using semipreparative HPLC with the compound identity verified by ^{1}H NMR spectra (Yamamoto *et al.*, 2002) measured on a 300 MHz Varian Mercury Plus NMR spectrometer (Palo Alto, CA, USA), at room temperature.

Results and Discussion

Tetracoumaroyl spermine was present in all tested commercially available chamomile tea bags commonly available on the Slovak market. Its content varied widely between 0.075–1.517 mg/g DW (Table I). Unlike in the work of Yamamoto *et al.* (2002) no considerable amount of 1*N*,5*N*,10*N*-tris[3-(4-hydroxyphenyl)-2-propenoyl]-1,5,10,14-tetraazatetradecane (triscoumaroyl spermine) was found in fresh methanol extracts. However, tetracoumaroyl spermine was quiet unstable in the extract, and several degradation products appeared after longer storage.

The basic factors determining the content of tetracoumaroyl spermine in the flower heads could be the plant developmental stage and environmental conditions (such as light, humidity, or availability of nutrients). Because tetracoumaroyl spermine is a new and promising natural medical

Table I. Tetracoumaroyl spermine content in commercially available tea bags.

Commercial producer – name of the product	Content (mg/g DW)[a]
Herbex s. r. o. – "Rumanček pravý"	1.517 ± 0.034
Fytopharma a. s. – "Rumančekový čaj"	1.251 ± 0.029
Leros s. r. o. – "Heřmánek"	0.755 ± 0.026
Agrokarpaty Plavnica s. r. o. – "Rumanček BIO"	0.484 ± 0.007
Baliarne obchodu Poprad a. s. – "Rumanček pravý"	0.467 ± 0.033
Klember a spol. s. r. o. – "Rumanček pravý"	0.229 ± 0.014
Belin Z. P. Ch. – "Rumianek"	0.075 ± 0.002

[a] Values are means of 3 replicates ± SE.

Table II. Tetracoumaroyl spermine content in main parts of flower heads.

Part	Content (mg/g DW)[a]
Receptacles	0.261 ± 0.012
Involucral bracts	0.394 ± 0.015
Ligulate flowers	0.297 ± 0.011
Tubular flowers	12.236 ± 0.483
Pollen	13.337 ± 0.679
Leaves	n.d.[b]
Roots	n.d.[b]

[a] Contents of tetracoumaroyl spermine extracted from 10 different plants.
[b] n.d., not detectable (< 0.05 mg/g DW).

compound (Yamamoto *et al.*, 2002), possible factors affecting the tetracoumaroyl spermine accumulation were examined.

Ten randomly selected flower heads in the 2nd phase of the tetraploid variety 'Lutea' were separated into receptacles, involucral bracts, tubular flowers, and ligulate flowers. Tetracoumaroyl spermine was present in all parts of the flower head, but its content in tubular flowers was about 40 times higher than in the rest of the inflorescence (Table II). Because the total mass of the tubular flowers is more than 60% of the whole inflorescence, approximately 98% of total tetracoumaroyl spermine occured in them. Within tubular flowers, tetracoumaroyl spermine is concentrated in pollen.

On the other hand, no detectable amount of this compound was found in the vegetative organs (roots and shoots). Some minor peaks of coumaroyl spermines with a lesser degree of coumaroylation were found in the extracts; their concentrations were neglible in the fresh extracts, but increased with time. So they are likely degradation products.

The floral development was divided into 5 different phases (see Material and Methods), and tetracoumaroyl spermine in one diploid ('Novbona') and one tetraploid ('Lutea') variety of chamomile was detected in each phase (Fig. 2). In the early stage of floral development, when the involucral bracts differentiate, but tubular and lin-

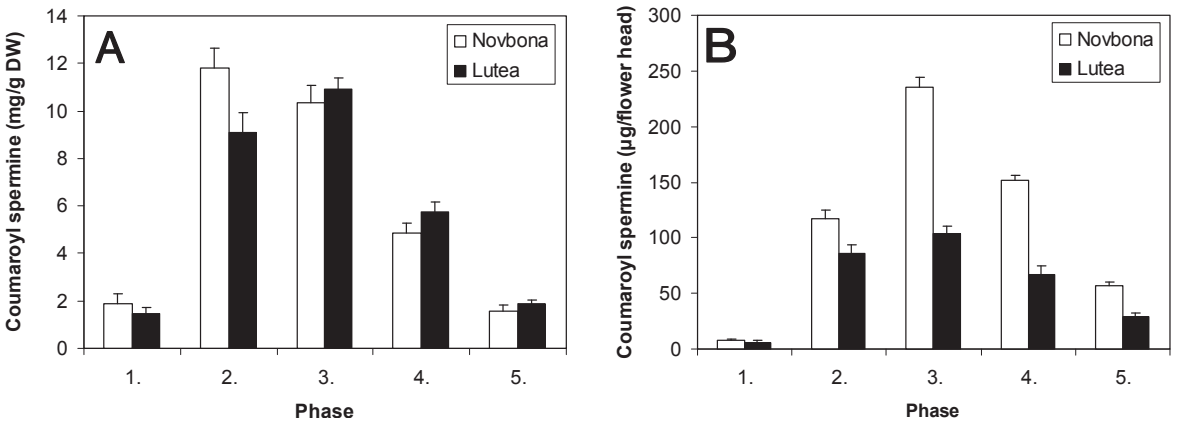

Fig. 2. Accumulation of tetracoumaroyl spermine during five phases of chamomile flower head development in the diploid (cv. 'Novbona') and tetraploid (cv. 'Lutea') cultivars. The results are means ± SE of 8 different plants (A) per dry weight and (B) per flower head, each with 3 separate replicates.

gulate flowers have just emerged and are hardly distinguishable, the tetracoumaroyl spermine level was very low. Later it increased rapidly and accumulated in the 3rd phase when the tubular flowers were beginning to open. In the 4th phase, its content decreased significantly and strongly dropped in the last phase. The reason is most likely the release of pollen, which contains a considerable amount of tetracoumaroyl spermine. Its level in pollen could be relatively stable, as was previously documented in bee-collected pollen from *Brassica campestris* (Williamson *et al.*, 2009).

No significant difference in the amount of tetracoumaroyl spermine, calculated on dry weight basis, was found between the diploid and tetraploid cultivars. The main difference between the cultivars was that the tetraploid cultivar had a higher mass of flower heads, therefore the content of tetracoumaroyl spermine calculated per head was also higher in each stage of flowering.

Phase 4 is the usual mature phase for harvesting. It could be supposed that the tetracoumaroyl spermine level, 0.49 mg/g fresh weight (FW) determined by Yamamoto *et al.* (2002), was in this phase of growth and thus this observation is in a good agreement with our results, taking into account that fresh weight is usually about 10 times higher than the corresponding dry weight. It may also be assumed that another reason for the relatively low amount of tetracoumaroyl spermine in the commercial drug is the late harvesting (closer to phase 5) and the strong release of pollen.

Concerning that tetracoumaroyl spermine is an N-containing compound, N-availability was expected to be an important environmental factor affecting its accumulation in the flowers. For this reason N-deficiency experiments were performed. Withdrawal of nitrogen from the previously N-sufficient medium resulted in nitrate exhaustion in the leaves of chamomile plants already just 10 days after the treatment (Fig. 3). The total N content in the leaves after 12 days on –N media

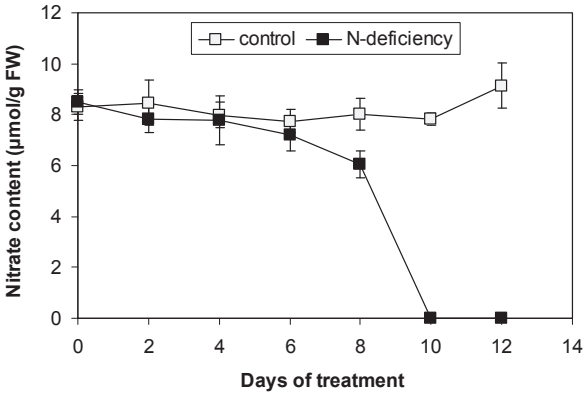

Fig. 3. Decrease of nitrate content in leaves of chamomile cv. 'Lutea' after its exposure to N-free Hoagland's solution (closed symbols), in comparison with control kept on the standard half-strength Hoagland's solution (open symbols). The results are means ± SE of 4 separate plants, each with 3 separate replicates. Error bars are not shown where they do not exceed dimensions of the symbol.

decreased to about 38% in comparison to +N-treated plants (Table III). Decrease of the total N content was also notable during the floral development, reaching 20% in fully developed flowers in the 4th phase, confirming the nitrogen shortage as well as re-distribution of nitrogen into the reproductive organs. The decrease in the levels of most of the individual free amino acids in N-deficient plants showed a similar pattern as the decrease of the total N content, being stronger in the leaves than in the flowers. The analysis was focused on the aromatic amino acids that are precursors of many secondary metabolites with aromatic ring structures, including flavonoids and coumarins that often make up a substantial amount of total dry weight of the inflorescens. The aromatic amino acids content (Phe, Tyr, and Trp) in the leaves decreased slightly and almost no decrease in their content was observed in flower heads (Table IV). The other free amino acids (such as Lys, Ala, Val,

Table III. Total nitrogen content in leaves and flower heads.

Material	Total amino N content (mg/g DW)[a]		Total protein content (mg/g FW)[a]	
	Control	N deficiency	Control	N deficiency
Leaves	52.4 ± 1.4	32.3 ± 1.2	13.16 ± 0.5	10.75 ± 0.5
Flower heads, 3rd phase	43.7 ± 1.6	37.4 ± 0.6	9.94 ± 0.4	8.60 ± 0.3
Flower heads, 4th phase	42.2 ± 2.0	34.0 ± 1.3	9.82 ± 0.5	8.01 ± 0.4

[a] Values are means of 6 replicates ± SE.

Table IV. Effect of nitrogen deficiency on contents of free amino acids in shoots and flower heads of 3rd and 4th phase of floral development.

Amino acid	Shoots		Flower heads, 3rd phase		Flower heads, 4th phase	
	Control[a]	N deficiency[a]	Control[a]	N deficiency[a]	Control[a]	N deficiency[a]
Phe	0.115 ± 0.019	0.108 ± 0.011	0.112 ± 0.006	0.120 ± 0.009	0.096 ± 0.008	0.099 ± 0.008
Tyr	0.040 ± 0.002	0.028 ± 0.001	0.027 ± 0.004	0.035 ± 0.001	0.026 ± 0.003	0.024 ± 0.002
Trp	0.254 ± 0.021	0.202 ± 0.024	0.154 ± 0.015	0.154 ± 0.016	0.189 ± 0.023	0.162 ± 0.029
Lys	0.351 ± 0.031	0.159 ± 0.019	0.256 ± 0.011	0.202 ± 0.013	0.267 ± 0.011	0.210 ± 0.007
Val	0.899 ± 0.057	0.484 ± 0.014	0.694 ± 0.033	0.534 ± 0.015	0.675 ± 0.020	0.508 ± 0.040
Ala	0.641 ± 0.040	0.267 ± 0.033	0.334 ± 0.016	0.303 ± 0.008	0.471 ± 0.032	0.382 ± 0.014
Leu	0.263 ± 0.034	0.191 ± 0.023	0.279 ± 0.022	0.223 ± 0.016	0.235 ± 0.011	0.202 ± 0.023

[a] Values are means (in μmol/g FW) of 4 replicates ± SE.

or Leu) were generally more affected, and their level decreased to a similar extent, especially in leaves.

Despite the overall nitrogen shortage, the accumulation of tetracoumaroyl spermine in the flower heads was found to be unchanged in the early stage of floral development, and surprisingly even a significant increase was determined in the 4th phase (Fig. 4). Similarly, its level also increased in the pollen, suggesting its high importance for pollen development. One putative function of tetracoumaroyl spermine could be in defence against pathogens, as was reported for tricoumaroyl spermidine from the pollen of *Quercus alba* (Walters *et al.*, 2001). Defensive functions of conjugated polyamines were suggested from several plant species, although they are based largely on correlation between their accumulation and pathogen resistance. The conjugated polyamines may regulate the free polyamines pool or act in their transport, and the role of free polyamines in plant defence is another area ripe for investigation (Walters, 2003). Coumaroyl spermines can also bind to cell-wall polysaccharides via the *p*-hydroxy group of the coumaric acid ring as well as to suberine and lignin in the process of cell-wall strengthening (Passardi *et al.*, 2004; MacAdam and Grabber, 2002).

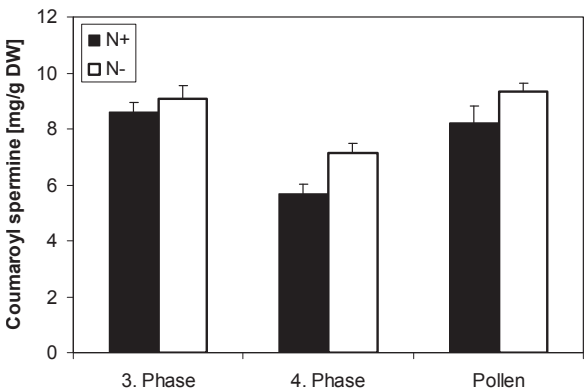

Fig. 4. Accumulation of tetracoumaroyl spermine in the 3rd and 4th phase of development of the flower heads and in pollen of chamomile cv. 'Lutea' at nitrogen deficiency in comparison with the N-sufficient control. The results are means ± SE of 8 different plants, each with 3 separate replicates.

It has been shown that tetracoumaroyl spermine is present mainly in the tubular flowers and reaches its maximum during the 3rd phase of flowering when the corollae of the tubular flowers in the anthodia start to open. The later decrease could be the result of the release of pollen that contains a considerable amount of tetracoumaroyl spermine. To achieve a high content of tetracoumaroyl spermine in the chamomile drug, earlier harvesting is needed. It is likely that tetracoumaroyl spermine has some important role during pollen development, and so despite the overall N-deficiency in the plants, tetracoumaroyl spermine is accumulated at the same or even higher rate than in flowers of the N-sufficient control.

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