Introduction of alkanet (*Alkanna tinctoria* (L.) Tausch), a traditional dye plant into cultivation

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Key words: alkanet, *Alkanna tinctoria*, dye plant, cultivation, introduction, germination, accumulation of active substances

Summary: As a part of the research project to establish natural sources of plant pigments, possibilities of introduction of *Alkanna tinctoria* (alkanet) into cultivation were studied.

As a result of the germination experiment, the relevance of 21 days' duration of germination procedure was proved. To get high germination rate alkanet fruits are proposed to pretreat by gibberellinic acid (GA3) in the concentration of 400 ppm, overnight before sowing. This method results approximately 50 % germination rate.

The morphological and production properties of alkanet roots are characterized during ontogenesis. Transplanted populations can be characterized by numerous, thick and heavy roots comparing to the spontaneous ones. Thus, seed sowing and transplantation proved to be an effective method for cultivation of the species.

According to our results it can be concluded that in cultivation the optimal harvesting time of roots is at the end of the second vegetation cycle, when the dry root mass of the individuals is about 10-20 g with 3,0-3,5 % accumulation level of active substances.

Considerable seasonal variability have been found influencing not only the root masses, but also the accumulation levels of alkannin derivatives. In a more humid vegetation cycle the root size and mass as well as the content of active substances are much higher.

Introduction

Alkanna tinctoria belonging to the family Boraginaceae is a protected medicinal and dye plant occurring mostly in open sand grassland communities of the Danube-Tisza Mid-Region of Hungary. To establish natural raw material of alkanet plant pigments, possibilities of introduction of Alkanna tinctoria (alkanet) into cultivation were studied.

Alkanet is an indigenous species in the south-eastern part of Europe and in Arabia. In Hungary it is a representative element of the submediterranean flora. *Alkanna tinctoria* can be characterized as a highly drought tolerant plant, it requires warmth, long duration of sunshine in the vegetation period and calciferous soils, poor in humus. Therefore, this species occurs on limestone rocks in the Mediterranean region or loose sandy stream deposits in Hungary. In the latter case, alkanet is a frequent constituent of the perennial open sand grasslands *(Festucetum vaginatae danubiale)* (*Horváth* et al, 1995).

Alkanet is a hemichryptophyte species flowering from April to May. Its roots are dark red, 15–20 cm long with a diameter of 1–3 cm, wearing a leaf rosette on the top with a lot of narrow, rigid hairy leaves.

The drug of alkanet *(Alkannae radix)* consists of the chopped blackish red roots of the plant. The dried roots are covered by a crust easily coming off, their inner part are yellowish white. The pigments can be found mainly on the surface of the crust *(Metcalf & Chalk,* 1957*)*, therefore the root is not suggested to wash before drying (*Lenchés*, 1993). Alkannae radix is official in many Pharmacopoeas (*Penso*, 1993). Its quality requirements are assumed in drug (*Anonym*, 1988) and food (*Anonym*, 1967) standards of Hungary as well as in twelve other European descriptions.

Alkannae radix contains dark-red naphthoquinone derivative pigments with biological activity, resins, tannins, waxes and pyrrolizidin alkaloids. Concerning the naphthoquinone group, alkannin is the most important aglicon, which rarely occurs naturally in free form. It is esterified by different acids, e.g. valerianic, isovalerianic, angelianic and dimethylacrilic acids. These compounds are grouped into lipophylic red pigments (*Papageorgiou*, 1978).

The pigments of the root are utilized for dyeing since the antiquity, known by the Greek and Roman textile craftsmen.

In Hungary the alkanet root was applied for marking the fur of lambs, which manifests itself in the the local Hungarian name of the species. However, the village people painted face, lips and nails as well as ointments and candies by this

Table 1 Seed pretreatment conditions applied during germination experiment of *Alkanna tinctoria*

Number	Treatments
Thermostate	
1	Control
2	Presoaked in warm water (40∞C)
3	GA3 200 ppm/24 hours
4	GA3 400 ppm/24 hours
5	GA3 600 ppm/24 hours
6	GA3 800 ppm/24 hours
7	GA3 1000 ppm/24 hours
Greenhouse	
8	Control
9	GA3 200 ppm/24 hours
10	GA3 400 ppm/24 hours
11	GA3 600 ppm/24 hours
12	GA3 800 ppm/24 hours
13	GA3 1000 ppm/24 hours

natural dye (*Járainé Komlódi*, 1990). Nowadays, alkanet is used mainly in the food industry as an official natural colourant of cheese and beverages.

The wound healing properties of *Alkanna tinctoria* is known, as well, since the creation of „The Greek Herbal of Dioscorides". It is effective against ekcema, acne, burns and piles. The alkannin derivatives possess the therapeutic activity which was proved in the course of the past twenty years. These pigments serve as basic materials of antibacterial, antiinflammatory and wound regenerating

Table 2 The most important data of weather conditions during vegetation periods of 1997 and 1998 (Budapest-Pestszentlőrinc)

	Average temperature, C°		Duration of sunshine, h		Total precipitation, mm	
Months	1997	1998	1997	1998	1997	1998
March	6.1	4.8	210	203	11	9
April	9.0	12.2	245	158	21	93
May	17.0	15.7	261	256	45	89
June	19.8	20.9	269	282	39	61
July	18.3	21.6	228	292	52	63
August	21.8	22.0	222	317	37	36
September	17.2	15.4	256	149	7	137

preparations. Antioxidant, adstringent and UV-protection features of these dyes pay important role in the relief as well. Moreover, in the case of alkannin–β,β–dimetilakrilate and alkannin acetate, the presence of the citotoxic activity was proved (*Papageorgiou*, 1978; *Papageorgiou*, 1980).

During the two years of investigations (1997–98) both laboratory and open field experiments were carried out. As a first step, the germination rate and propagation possibilities of alkanet were studied. To get an economical culture, the effects of age, growing season, propagation methods and ontogenetical phases on morphological and production biological properties were analysed, afterwards. The next purpose of our investigation was to optimize the harvest time to get a maximum of biomass and active agents.

Material and methods

Germination experiments

The fruits of alkanet were collected in 1996 from natural populations. Because of the lack of literature data, at first the average seed production of wild growing individuals and the thousand seed weight had to be determined.

The germination experiment was carried out in the laboratory of Department of Medicinal and Aromatic Plants (SZI University, Budapest) as well as under greenhouse conditions. Practically, the „seed" name will be used afterwards instead of the botanically perfect „fruit".

The seeds were germinated in thermostate (Hotpack Illuminated Chambers 352622 25°C/day, 20°C/night) in Petri dishes. As specific standards for the germination procedure of alkanet could not been found in the literature, we used the actual Hungarian Standard for Borago officinalis belonging to the same plant family (*Anonym*, 1992). In the laboratory experiments 50 seeds were put into Petri dishes on the surface of filter paper in three repetitions at each treatment listed in *Table 1*. The conditions of greenhouse test were as follows: the pretreated seeds were sown into propagation pots filled with general propagation medium, by the 0.5 cm depth of sowing. During germination and raising of seedlings, moderate greenhouse circumstances have been kept.

Open field experiments

The open field experiments were carried out in the Research Station of SZI University Soroksár (Budapest). Two types of plant material were compared. One of them was represented by indigenous *Alkanna tinctoria* populations spred spontaneously among the advantageous ecological conditions of the Resarch Station (it is situated close to sandy deposit of Danube river). These plants were two and three years old at the time of the two years of investigations.

The second group of plants was established by seed sowing and transplantation in 1996 and 1997. The seeds were sown into propagation pots in greenhouse in the middle of March in both years. The seedlings were picked in April and transplanted into open field in early June by using plant density of 50x40 cm. The care of plants involved the mechanical weed control and the irrigation. In the middle of June, stems holding the ripe fruits were cut leaving the leaf rosettes on the plants.

In the course of the experiments (1996–1998) the morphological and production biological characteristics as well as the accumulation processes of active substances were

Table 3 Morphological and production biological characteristics of *Alkanna tinctoria* investigated by phenophases

Sampling times	Morphological characteristics	Production biological characteristics
1. March 20–30 (start of sprouting) 2. April 10–20 (budding) 3. May 1–10 (full flowering) 4. June 1–10 (fruit ripening) 5. August 1–10 6. October 1–10 (end of vegetation cycle)	Leaf rosette size in diameter, cm Average leaf length, cm Average leaf width, cm Root length, cm Root thickness, cm	Dry root weight, g/plant Content of active substances, g/100g Yield of active substances, g/m^2

Table 4. Morpho-phenological properties of second year old spontaneous (S) and transplanted (T) *Alkanna tinctoria* populations

Sampling times	Leaf rosette size in diameter, cm		Leaf length, cm		Leaf width, cm		Root length, cm		Root thickness, cm	
	S*	T**	S	T	S	T	S	T	S	T
1	13.60	19.00	1.92	1.80	0.34	0.30	20.60	13.00	1.08	3.00
2	25.30	35.00	2.95	2.00	0.41	0.50	14.10	18.00	1.37	3.00
3	42.20	46.00	2.42	2.10	0.45	0.50	16.25	18.00	1.50	3.50
4	51.10	51.00	7.65	5.50	0.72	0.50	19.25	22.00	1.09	2.90
5	42.10	48.00	10.48	7.00	0.74	0.80	16.85	17.00	1.06	3.00
6	27.90	29.00	8.90	7.50	0.72	1.00	15.70	9.00	1.13	3.00
$LSD_{5\%sampling\ time}$	9.84	12.57	1.57	1.63	0.20	0.28	4.94	10.55	0.54	0.80
$LSD_{5\%propagation}$	3.20		0.53		0.06		3.85		1.50	
$LSD_{5\%\ sampling\ time}$	5.62		0.92		0.12		6.67		2.59	

*S= spontaneous
**T= transplanted

Table 5. Morphological characteristics of transplanted second year old Alkanna tinctoria populations in different growing seasons (1997 and 1998)

Sampling times	Leaf rosette size in diameter, cm		Leaf length, cm		Leaf width, cm		Root length, cm		Root thickness, cm	
	1997	1998	1997	1998	1997	1998	1997	1998	1997	1998
1	19.00	23.20	1.80	1.09	0.30	0.32	13.00	17.10	3.00	2.58
2	35.00	25.50	2.00	2.11	0.50	0.50	18.00	17.30	3.00	2.87
3	46.00	41.90	2.10	2.31	0.50	0.53	18.00	19.60	3.50	3.23
4	51.00	74.40	5.50	1.85	0.50	0.63	22.00	18.30	2.90	3.50
5	48.00	47.70	7.00	10.00	0.80	1.11	17.00	16.30	3.00	3.15
6	29.00	43.00	7.50	8.40	1.00	1.03	9.00	38.40	3.00	4.10
$LSD_{5\%sampling\ time}$	12.57	14.14	1.63	1.37	0.28	0.32	10.55	16.52	0.80	1.11
$**LSD_{5\%\ growing\ season}$	4.91		0.65		0.09		5.47		0.34	
$**LSD_{5\%sampling\ time}$	8.51		1.12		0.16		9.46		0.59	

*LSD obtained by one-way analysis of variance, yearly
**LSD obtained by multifactor analysis of variance

studied in both indigenous and transplanted populations. Ten randomly chosen individuals have been measured at each sampling time, according to *Table 3*. The effects of seed sowing and transplantation method on second year-old populations (1997), duration of vegetation cycle and the growing seasons (1997 and 1998) were analized.

Characterization of ecological conditions

The soil conditions of the experimental plots were determined by the Central Laboratory, Faculty of Food Sciences, SZIU in 1998. Based on the results the calciferous loose sandy soil of the experimental plots is characterised by fairly good K_2O (378 mg/kg) and P_2O_5 (345 mg/kg) content and by poor accessible N (1,65 mg/kg) supply.

The weather conditions of the growing seasons are shown in *Table 2.* using data of Budapest-Pestlőrinc Meteorological Station.

Analysis of active components

Laboratory method developed by us recently (*Pluhár* et al, 2000) was used for the extraction of active substances. It was made by supercritical fluid extraction (SFE). The extraction pressure of 200 bar, temperature of 50°C, extraction time of 30 minutes and proportion of n-hexane modifier of 10 % were applied. The active substance content meant the total extracted material, expressed in weight % (g extract/100g of dry matter). This value comprised mainly the naphtoquinon derivatives being present in the homogenized root powder.

Methods of statistical evaluation

To evaluate germination and production biological characters affected by different treatments, vegetation cycle, transplantation, weather conditions, etc. one-way and multifactor analysis of variance were used applying Statgraphics 5.1 software.

Results and discussion

Characterization of seed yield of wild growing populations

It was proved by us that the wild growing individuals produce about 7–8 g seeds per plant. The value of the thousand seed weight is as much as 2.5 –3.0 g.

Germination biological findings

According to the germination test, the first germs of control and soaked seeds appeared on the 7th day, while the seeds pretreated by different concentrations of gibberellinic acid had begun their germination 3 days earlier. Each seedlings wore a reddish ring on the radicula as a first sign of the appearance of plant pigments in this early age. When comparing the effects of the different pretreatments expressed in germination power on the 7th day, there was no significant difference between them *(Figure 1)*.

At the end of the germination period, on the 21st day, there were significant differences among the germination of control and soaked, as well as the pretreated seeds. The effect of different GA_3 concentrations shows an optimum curve, where the maximum (26 per cent of seeds germination) occurs at 600 ppm. This meant no important deviation from the treatments of 200, 400 and 800 ppm , respectively.

In the greenhouse experiment, however, the duration of germination was longer because of the small germs had to grow through the soil layer, the tendency of germination rate is much similar to that of the laboratory test *(Figure 2)*. Nevertheless, in this experiment the values were approximately the double, regarding the corresponding treatments in the thermostate. In the greenhouse test, the seeds pretreated by 400 ppm GA_3 showed the best and significant result (54%). It does mean practically, that providing the optimum pretreatment (400 ppm GA_3 overnight before sowing) about the half of the sown seeds germinates and results seedlings.

The germination rate reached by the above mentioned treatments is relatively low, but it is a characteristic features of wild growing species, generally. However, in harmony with great number of observations the weather of the vegetation cycle, the ripening stage of fruits, the length of storage may also influence the germination rate. For instance in the case of related species *Borago officinalis* an after-ripening can be observed (*Tóth*, 1999). In the future, biological circumstances, which may increase the germination rate of *Alkanna tinctoria* has to be studied.

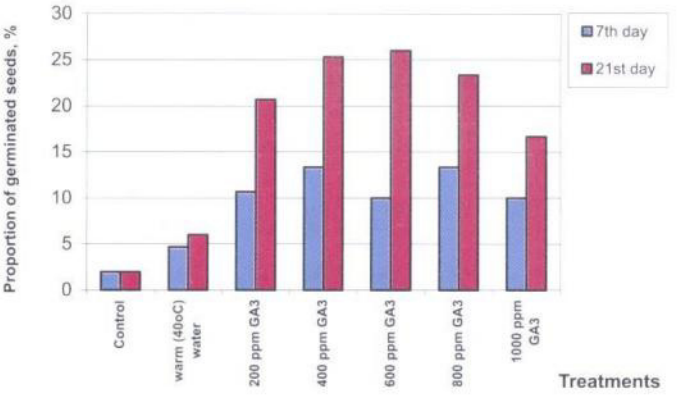

Figure 1 The values of germination power (7th day) and germination rate (21st day) of *Alkanna tinctoria* seeds affected by different pretreatments
($LSD_{5\%germ.\ power}$=12.35%, $LSD_{5\%germ.rate}$=14.34%)

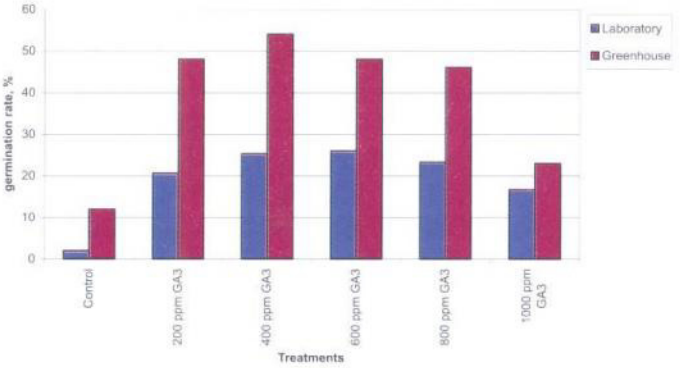

Figure 2 The values of germination rate of *Alkanna tinctoria* seeds affected by different pretreatments in laboratory and greenhouse tests
($LSD_{5\%greenhouse}$=4.58%, $LSD_{5\%laboratory\text{-}greenhouse}$=10.17%)

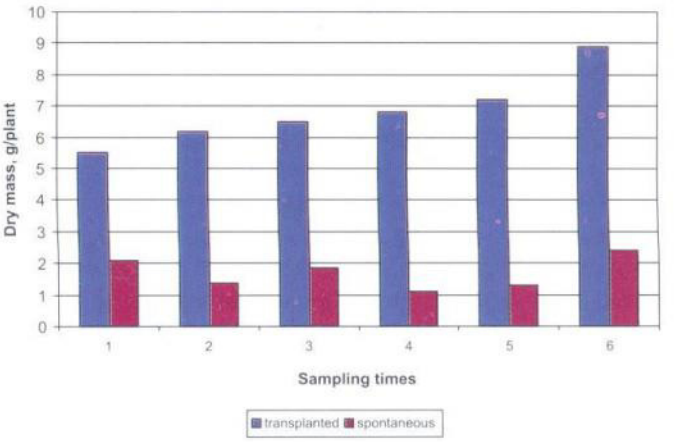

Figure 3 Changes in dry root mass during the vegetation cycle in second year-old spontaneous and transplanted alkanet populations

Differences between spontaneous and transplanted alkanet populations

The main morpho-phenological features of indigenous Alkanna tinctoria populations and plants propagated by seed sowing followed by transplantation is shown in *Table 4*.

Regardless of propagation method, the aerial parts

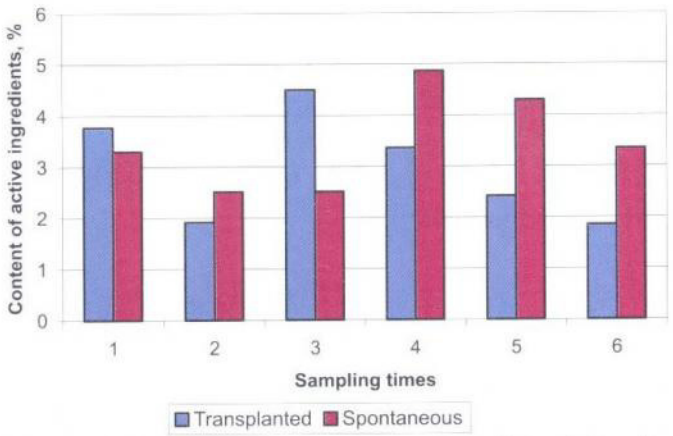

Figure 4 Changes in content of active ingredients during the vegetation cycle in second year-old spontaneous and transplanted alkanet populations

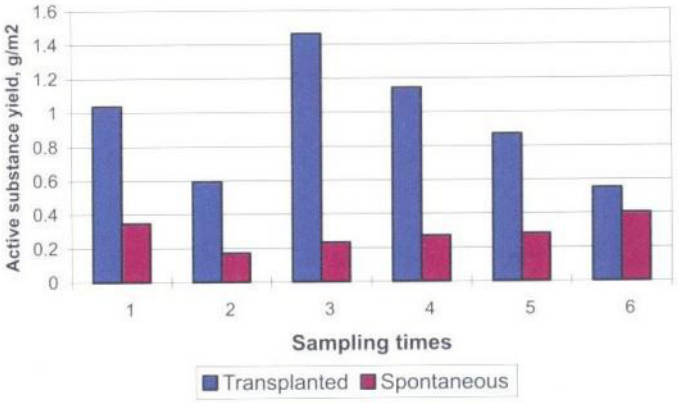

Figure 5 Changes in active substance yields during the vegetation cycle of 1997 in second year-old spontaneous and transplanted *Alkanna tinctoria* populations

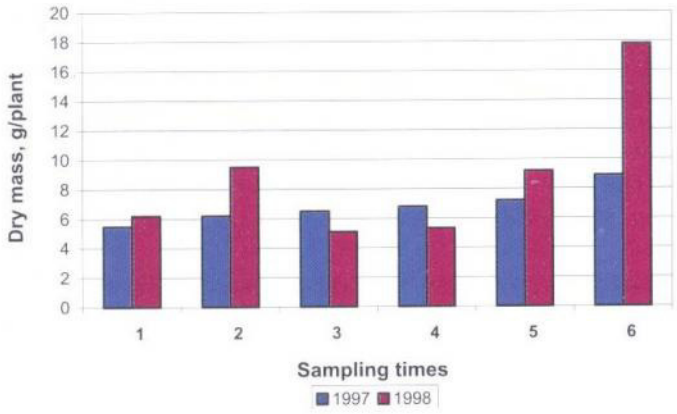

Figure 6 Changes in dry root mass during 1997 and 1998 in second year-old transplanted alkanet populations

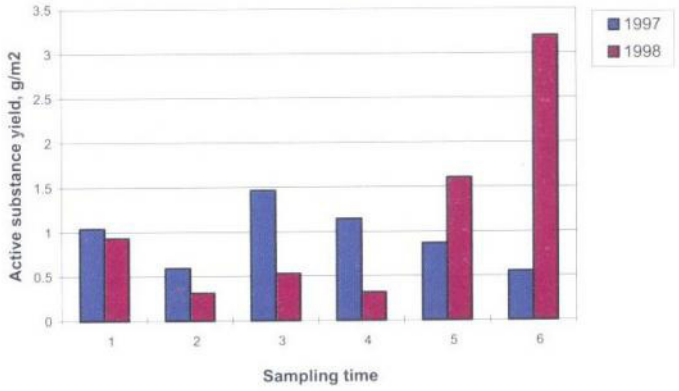

Figure 7 Changes in active substance yields during 1997 and 1998 in second year-old transplanted *Alkanna tinctoria* populations

developed in the similar way. The width of leaf rosettes reaches the maximum value during fruit ripening in both second year-old indigenous and transplanted populations. After cutting the shoots of ripe fruits in June, the leaves remaining started to grow, therefore the values of leaf size increased afterwards.

The spontaneous population of Alkanna tinctoria can be characterized by overall heterogeneity of the morphological properties, while transplanted ones showed much more uniformity. Transplanted plants grew larger leaf rosettes and significantly more flowering shoots, however, the spontaneous plants held less but longer leaves. According to the statistical evaluation the size of these plant organs are influenced by phenophases, too. In the case of both transplanted and indigenous populations the effect of phenophases was justified by one-way analysis of variance. Using multifactor analysis regarding propagation and ontogenetical phases at the same time effects of both factors proved to be significant.

Considerable divergences were found between the roots of spontaneous and transplanted plants of the same age considering the maximum root length and root thickness values. The spontaneous plants had less, but longer roots, while in the case of picked and transplanted populations larger roots with high number of side roots were observed. Altogether, the maximum of root thickness were measured at the transplanted plants at the time of flowering. The decrease of root length and thickness after the generative phases can be explained by the withering and elimination of the aged large roots parallelly with the degradation of aerial parts. The relatively dry summer of 1997 accelerated the appearance of this phenomenon.

There were significant divergences in root yield of spontaneous and transplanted plants, too, which can not be explained by differences of root morphology *(Figure 3)*. Until the end of vegetation period, parallelly by the concentration of dry matter content and formation of new side roots, the total dry mass of root increased continuously, as a general rule. Without any respect to phenological phases, transplanted populations possessed higher root yields when comparing them to the spontaneous ones.

Higher maximum content of *active substances* was reached by spontaneous *Alkanna* populations than the transplanted ones of the same age *(Figure 4)*. In the case of spontaneous populations the maximum of accumulation was determined in the phase of fruit ripening, while in the transplanted ones it occurs during flowering. Active substance yields of transplanted populations exceeded

considerably the respective values of spontaneous ones in each ontogenetical phases, though, with some variations *(Figure 5)*.

Effect of growing season on transplanted populations

It was proved by us that the morphological traits of *Alkanna tinctoria* are modified by seasonal differences, too. The plants grew bigger leaf rosettes in 1998, which can be explained by the higher amount of precipitation during fruit development *(Table 2, Table 5)*. However, the leaf characteristics were mainly affected by phenological phases.

The *root* size and development was determined by frequent autumn rainfalls too, which was rather characteristic in 1998. The length of main root (38.4 cm) and the root thickness (4.1 cm) reached its maximum at the end of the vegetation period. It can be explained by the outstanding amount of precipitation fallen in September of 1998, which may resulted some kind of plant regenaration *(Table 5)*.

The maximum of *dry root yield* has been measured at the end of both vegetation periods. In 1997 a continuous but small scale increase was experienced, however, significant changes were observed in the vegetation cycle of 1998 *(Figure 6)*. In the latter case, during the generative phases (flowering and fruit ripening) the drug yield has decreased. Then a period of considerable increase followed, where the values detected in autumn exceeded approximately twofold those of the previous phases and year, respectively.

The extract content as well as the the yield of active substance of *Alkannae radix* also have been proved to show variability affected by the year *(Figure 7)*. These parameters were also affected by precipitation and root development. In 1997 and 1998 they followed the same tendencies as the root length and weight. The extract content varied between 1.9 % and 4.5 % in 1997 or 0.7 % and 3.6 % in 1998, while the values of active substance yields changed between 0.6 g/m^2 and 1.5 g/m^2 in 1997 or 0.3 g/m^2 and 3.2 g/m^2 in 1998. During fruit ripening a decline could be experienced regarding these properties. Later on, as a result of the autumn precipitation and dry matter increase in 1998, the roots regain their weight and active agent content. Otherwise, in 1997, these values remain low stabilizing the summer decrease.

Possibly, in an arid year (1997), which provide normal ecological circumstances to alkanet, the maximum of active ingredients can be determined during flowering as the pathways of secondary metabolism are able to operate and produce the alkannins. Among more humid conditions (1998), however, these functions are probably retarded and the accumulation drags on till the autumn.

Acknowledgement

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