Breeding support for *Valeriana officinalis* L. s.l.:

Root structure, localization of value-determining secondary compounds and mating behavior at open pollination

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Abstract

Valerian (*Valeriana officinalis* L. s.l.) is an important medicinal plant, which is used for insomnia and restlessness. Preparations for treating of such indications are produced from the dried rootstock (roots and rhizome). The raw material comes invariably from field cultivation. Due to the very fine, carpet-like root structure, a high proportion of soil is attached to the root system after the harvest, which requires an extensive post-harvest treatment. Losses of root-yield and important secondary compounds are the consequences. Therefore, a breeding project was set up to develop varieties with coarser root structure and acceptable secondary compound contents by selection- and cross-breeding.

The aim of this thesis was to investigate two fundamental aspects in order to support valerian breeding:

- Root structure analyses and the localization of secondary compounds within the root system
- Mating behavior under conditions of open pollination in the field

Knowledge about the relationship between root structure and secondary compounds contents is of central importance for the breeding project. For instance, a decrease of secondary compound contents with increasing root thickness would be disadvantageous for the primary breeding goal.

Two techniques were developed and applied to localize secondary compounds in different parts of the root system of four cloned valerian genotypes. An analytical determination of the valerenic acids and the essential oil contents was performed by HPLC and water-distillation, respectively. The horizontal distribution of the essential oil droplets within root cross-sections was determined by a fluorescence-microscopic image-analysis.

Based on the analytical investigations on the sesquiterpene acids (sum of valerenic acid and acetoxyvalerenic acid) and essential oil contents, the adventitious roots showed 25 to 30 % higher contents than the lateral roots, and these in turn had a 25 to 30 % higher contents compared to the rhizomes. Differences between four root diameter fractions were not detectable for any secondary compound. The image analysis showed that 43 % of the detected oil droplets were located in the root cortex (parenchyma) close to the root surface (epidermis). The remaining 57 % were detected in the subjacent root cortex (inner parenchyma). The central cylinder was free of essential oil droplets. Differences in oil droplet distribution and density between the genotypes, the root thickness and the harvesting depth are detectable. In summary, the results indicate that a coarser root structure did not lead to a decrease of important secondary compounds.

The second aim of this thesis dealt with the determination of the outcrossing rate under conditions of open pollination. Cross-pollination is described as the naturally pollination system of valerian, however self-pollination cannot be excluded. Within the offspring from open pollinated and random mated (panmixia) valerian plants, the proportion of descendants, who were generated by cross-pollination, was determined by marker based analyses (AFLP). The cross-pollination was accepted, if the reference marker of the crossing partner was detected. The determined proportion of cross-

pollination ranged from 76.5 to 97.7 %. Thus, the predominant tendency of the valerian breeding material to outbreeding was confirmed.

Key words: sesquiterpene acids, valerenic acid, essential oil, image analysis, molecular markers, AFLP

Zusammenfassung

Baldrian (*Valeriana officinalis* L. s.l.) ist eine bedeutende Arzneipflanze, welche bei Schlafstörungen und Unruhezuständen angewendet wird. Präparate zur Behandlung solcher Indikationen werden aus dem getrockneten Wurzelstock (Wurzeln und Rhizom) hergestellt. Das Rohmaterial hierzu stammt heutzutage ausnahmslos aus dem Feldanbau. Wegen der sehr feingliedrigen, teppichartigen Wurzelstruktur wird während und nach der Ernte ein hoher Erdanteil im Wurzelstock festgehalten, was zu einer aufwändigen und zu einer wenig wurzelschonenden Nacherntebehandlung führen kann. Ertrags- und Inhaltsstoffverluste sind die Folge. Deshalb wurde ein Züchtungsprojekt gestartet, das durch Auslese- und Kreuzungszüchtung Sorten mit grober Wurzelstruktur und akzeptablen Inhaltsstoffgehalten entwickeln sollte.

Die vorliegende Dissertation hatte zum Ziel, zwei grundlegende, für die Züchtungsarbeit wichtige Aspekte zu untersuchen:

- Den Zusammenhang von Wurzelstruktur und Inhaltstoffen, sowie
- Das Kreuzungsverhalten bei freier Abblüte.

Kenntnisse über den Zusammenhang von Wurzelstruktur und Inhaltstoffgehalten sind von zentraler Bedeutung für das Zuchtprojekt. Verringern sich beispielsweise die Inhaltsstoffgehalte mit zunehmender Wurzeldicke, wäre dies zum Erreichen des primären Zuchtziels von Nachteil.

Zur Lokalisierung der Inhaltsstoffe wurden zwei Verfahren entwickelt und bei verschiedenen Baldriangenotypen und unterschiedlichen Teilen des Wurzelsystems angewendet. Eine analytische Bestimmung des Gehalts an Valerensäuren und an ätherischem Öl erfolgte mittels HPLC und Wasserdestillation. Die horizontale Verteilung des ätherischen Öls im Wurzelquerschnitt wurde mittels einer fluoreszenzmikroskopischen Bildanalyse untersucht.

Die analytischen Untersuchungen zeigten, dass die Gehalte an Valerensäuren und ätherischem Öl in den Adventivwurzeln 25 bis 30 % höher waren als in den Seitenwurzeln und deren Gehalte wiederum 25 bis 30 % höher als die der Rhizome. Unterschiede zwischen vier Wurzeldurchmesser-Fraktionen waren nicht nachweisbar. Die Bildanalyse zeigte, dass 43% der detektierten Öltröpfchen in der nahe der Wurzeloberfläche (Epidermis) befindenden Wurzelrinde (Parenchym) zu finden waren. Die restlichen 57 % befanden sich im darunterliegenden Rindengewebe (innerem Parenchym). Der Zentralzylinder war frei von Öltröpfchen. Unterschiede in der Öltröpfchenverteilung und -dichte zwischen den Genotypen, der Wurzeldurchmesser und der Erntetiefe waren feststellbar. Zusammenfassend zeigen die Ergebnisse, dass eine grobe Wurzelstruktur nicht zu einer Abnahme wichtiger Sekundärverbindungen führte.

Das zweite Ziel dieser Arbeit beschäftigte sich mit der Bestimmung der Auskreuzungsrate unter freier Abblüte. Die Fremdbestäubung wird als das natürliche Bestäubungssystem des Baldrians beschrieben, jedoch kann Selbstbestäubung nicht ausgeschlossen werden.

Innerhalb der Nachkommen von panmiktisch bestäubten Baldrianpopulationen wurde der Anteil der aus Fremdbestäubung entstandenen Nachkommen, mittels markerbasiert Analysemethoden (AFLP) bestimmt. Eine Fremdbefruchtung war gegeben, wenn der Referenzmarker des Kreuzungspartners gefunden wurde. Der ermittelte Anteil an Fremdbestäubung lag zwischen 76,5 und 97,7 %. Die Präferenz des Baldrian Zuchtmaterials zur Fremdbefruchtung, wurde somit bestätigt.

Schlüsselwörter: Sesquiterpensäuren, Valerensäure, ätherisches Öl, Bildanalyse, molekulare Marker, AFLP

Table of Contents

List of Abbreviations / Abkürzungsverzeichnis

1n	description of a gametic cell
1x, 2x, etc.	chromosome number: haploid, diploid, etc.
2n	description of a somatic cell
AFLP	amplified length polymorphism-analysis
AR	adventitous roots
BAP	benzylaminopurin
BMEL	Bundesministerium für Ernährung und Landwirtschaft
CaO	calcium oxide
CE	cloned elite plants
DNA	desoxyribonucleic acid
FNR	Fachagentur Nachwachsende Rohstoffe e.V.
FPA-detector	focal plane array detector
FPA-solution	formaldehyde-propionic acid-ethanol-solution
FTIR	fourier-transform infrared
GABA	gamma-aminobutyric acid
GCA	general combining ability
HPLC	high performance liquid chromatography
HZ	root horizon
IMS	mass spectrometry imaging
ITS	internal transcribed spacer
K_2O	potassium oxide
KAMEL	Joint research project 'Improving the international competitiveness of the German production of medicinal and aromatic plants based on breeding and agro-technological optimizations of chamomile, valerian and lemon balm as model crops'.
KOH	potassium hydroxide
MALDI	matrix-assisted laser desorption ionization
L	location
LfL	Bayerische Landesanstalt für Landwirtschaft
LR	lateral roots
MgO	magnesium oxide
MPH	mid parent heterosis
MSL	mean sea level
N	nitrogen
OCR	outcrossing rate
P_2O_5	phosphorus pentoxide
PC	pair-combinations
rDNA	ribosomal DNA
resp.	respectively
RF	root thickness fraction, root diameter fraction
RI	rhizomes
SCA	specific combining ability
TK	thick rooted
TN	thin rooted

1. General Introduction of Valerian (*Valeriana officinalis* L. s.l.) and Valerian Breeding

Parts of the following chapter will be a part of the chapter ***Valerian, Valeriana officinalis L. s.l.*** of the book series ***Handbook of Plant Breeding*** (series editors Rajcan I. and Vollmann J.), volume ***Medicinal, Aromatic and Stimulant Plants*** **(**volume editors Novak J. and Blüthner W-D).

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1 Botany

Valeriana officinalis L., the common valerian, has a long application history in European folk medicine, which is also illustrated by the multitude of commonly used names, e.g., all heal, capons's tail, cat's love, garden heliotrope (Dweck 1997). The origin of the botanical name can be traced back to the Roman province Valeria in Pannonia, the region west of the Balaton (Hungary). The etymological description of the Latin *valere* (be healthy, be strong) was introduced at a later time (Mayer 2003).

1.1 Taxonomy

Valeriana is the eponymous genus of the family Valerianaceae and comprises approximately 150 to 350 species (Dweck, 1997; Bell and Donoghue, 2005). Recent studies have discussed the classification of Valeriana within the Caprifoliaceae family (APG, 2009). The systematic classification up to the genus category is clear (Table 1); however, the taxonomy within the *Valeriana officinalis* aggregate (agg.) has not yet been clarified (Buttler et al., 2008). Concerning the difficult taxonomic order, the different taxons are classified in different studies as species, subspecies or varieties. A frequently used classification is the basic taxonomic order of the *Valeriana officinalis* agg. resulting from the investigations of Skalinska (1947), Walther (1949), Titz (1969), Titz and Titz (1981; 1982; 1979), Titz et al. (1983) and Titz (1984).

Table 1. Classification of *Valeriana* according to Kirschner and Raab-Straube (2017+)

Category	Classification
Regnum	Plantae
Division	Tracheophyta
Subdivision	Spermatophytina
Class	Magnoliopsida
Superorder	Asteranae
Order	Dipsacales
Family	Valerianaceae Batsch
Genus	Valeriana L.

For valerian in Central Europe, Walther (1949) classified the five species, *V. exaltata*, *V. collina, V. pratensis*, *V. procurrens* and *V. sambucifolia,* by including morphological, chorological and chromosomal aspects. The main morphological characteristics for differentiation are leaf morphological traits and runner formation. In principle, Titz (1984) uses the same classification for the valerian in Germany/Bavaria but defined them as 'types' instead of species. Keller (1973) described for Switzerland the three subspecies *V. officinalis* L. ssp. *exaltata*, *V. officinalis* L. ssp. *collina* and *V. officinalis* L. ssp. *procurrens*.

For valerian in the Czech Republic, Holub and Kirschner (1997) differentiated among the species V. *officinalis L.*, *V. stolonifera* CZERN. and *V. excelsa POIR.* and allocated further subtypes to each of them. Buttler et al. (2008) applied this concept to the German flora and substituted

V. stolonifera CZERN. with *V. pratensis* DIERBACH. A recently published study showed that more ecologically and morphologically distinct groups of *V. pratensis* can be differentiated in Southern Germany (Gregor et al., 2016). Thereby, the informally recognized 'Frankonia-Type' of Titz (1984) has now been formally described and indicated as *V. pratensis* subsp. *franconica* Meierott and T. Gregor. Table 2 shows the current accepted taxonomy of the *Valeriana officinalis* agg.

The taxonomy is complex, and the currently available results are difficult to interpret. As it is not completely clear, on which the Central European species or subspecies the medically used valerian be ascribe the appendix *sensu lato* (s.l.) is often used. In "the broad sense", the whole *Valeriana officinalis* agg. is addressed in this case.

Table 2. Taxa of the *Valeriana officinalis* agg. included in the genus *Valeriana* L. according to Kirschner and Raab-Straube (2017+)

Species	**Subspecies**
Valeriana armena P. A. Smirn.	
	Valeriana armena P. Smirn. subsp. *armena*
	Valeriana armena subsp. *grossheimii* (Vorosch.) Vorosch.
Valeriana colchica Utkin	
Valeriana excelsa Poir.	
	Valeriana excelsa Poir. subsp. *excelsa*
	Valeriana excelsa subsp. *salina* (Pleijel) Hiitonen
	Valeriana excelsa subsp. *sambucifolia* (Pohl) Holub
	Valeriana excelsa subsp. *versifolia* (Brügger) Buttler & al.
Valeriana hispidula Boiss.	
Valeriana officinalis L.	
	Valeriana officinalis L. subsp. *officinalis*
	Valeriana officinalis subsp. *nemorensis* (B. Turk) F. Martini & Soldano
Valeriana pratensis Dierb.	
	Valeriana pratensis Dierb. subsp. *pratensis*
	Valeriana pratensis subsp. *franconica* Meierott & T. Gregor
Valeriana rossica P. A. Smirn.	
Valeriana stolonifera Czern.	
	Valeriana stolonifera Czern. subsp. *stolonifera*
	Valeriana stolonifera subsp. *angustifolia* Soó
*Valeriana wolg*ensis Kazak.	

1.2 Origin and Distribution

The species complex of *Valeriana officinalis* L. s.l. is characterized by enormous variability and can be divided into numerous subspecies with partially limited distribution areas. Species overlaps exist. The natural occurrence of the species complex is located in the temperate and boreal zone of Europe and Asia (Figure 1-A). The natural distribution areas are predominantly located between latitudes of 30° and 70° (Meusel and Jäger, 1965; 1978; 1992). In Germany, valerian is naturally present mainly in the southern and eastern regions (Figure 1-B). *Valeriana officinalis* L. s.l. grows mainly on fresh to moist habitats, e.g., moist and sparse deciduous forests, ditches, banks, shrubberies and meadows. Due to its frost tolerance, valerian can still be found in mountain regions up to altitudes of 1.800 m MSL (Heuberger et al., 2012a).

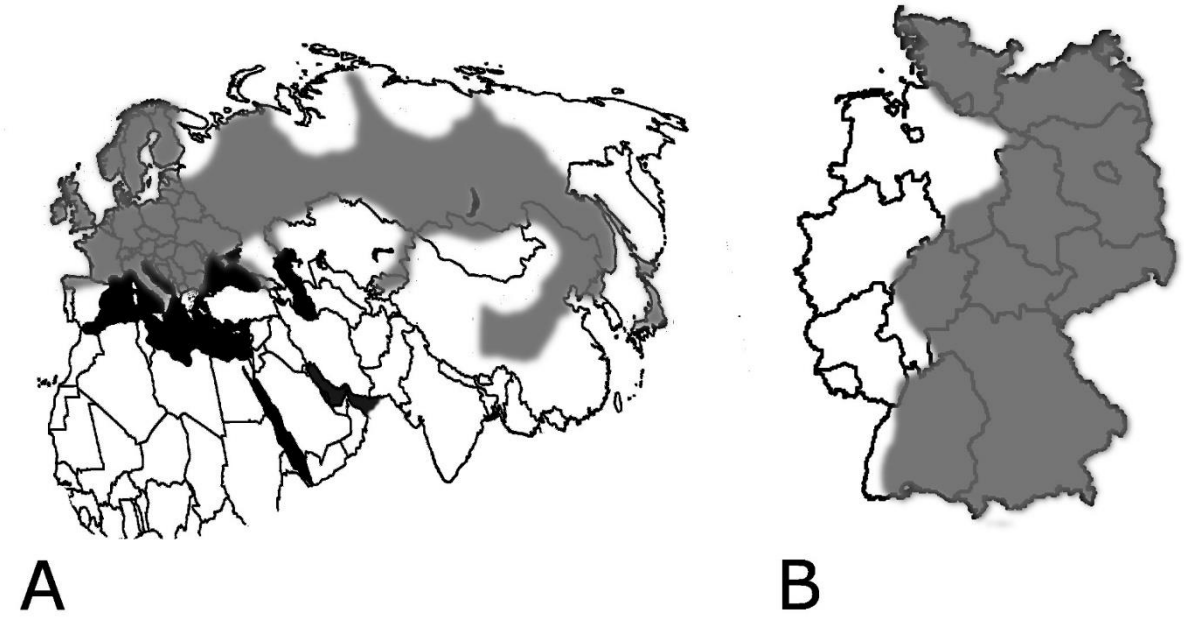

Fig. 1. The natural and predominant occurrence of the species complex of *Valeriana officinalis* L. s.l. in Europe and Asia (A) and specifically in Germany (B). (Data source: (Meusel and Jäger, 1965; 1978; 1992; BfN, 2017))

1.3 Cytology and Molecular Biology Aspects

1.3.1 Chromosome Number

Valeriana officinalis L. s.l. presents a basic chromosome number of $1n = 1x = 7$. The number of chromosomes is one important characteristic used to identify the different taxa. Early taxonomic investigations were often based on this number. The ploidy levels are compatible with the taxonomic classification of Walther (1949), Titz (1969), Holub and Kirschner (1997).

Mainly di-, tetra- and octoploid valerians are found in nature, but Hidalgo and Vallès (2012) and Heuberger et al. (2012b) also described natural hexaploid cytotypes. Up to the present time, the origination of the cytotypes remains unclear. Recently, an attempt was taken to investigate and clarify the origin of the polyploid-complex by genome analysis, two independent research groups genotyped

di-, tetra- and octoploid plants by amplified length polymorphism-analysis (AFLP) (Fischer, 2012; Heuberger et al., 2012b).

In both studies, the two-dimensional main component analyses showed that each of the three ploidy levels built a separate cluster. The genetic similarity was higher within compared with between the cytotypes, but single overlaps were observed. Heuberger et al. (2012b) showed that some accessions formed subclusters within the tetraploid cluster, but the accessions could be genetically curtailed because of breeding processes in the past. The high degree of consensus indicates a long-term development with limited gene flow between the cytotypes.

To verify the origin of the cytotypes, other methods are more suitable. The DNA sequence of the nuclear internal transcribed spacer-region (ITS-region) is a nonfunctional, tandem-like repeated DNA sequence located between nuclear and ribosomal DNA (rDNA). ITS-sequences have a high degree of polymorphism and are often used for genealogical studies and for identifying genetic variability. If a mutation occurs among the tandem-like sequences in the ITS-region, by molecular processes called "concerted evolution", all other tandem sequences will be replaced through this mutated sequence, or the mutated sequence will again become lost (Liao, 1999). In autopolyploid organisms, this process can affect all homologous chromosomes (multivalents). In the case of strict allopolyploids, "concerted evolution" occurs mostly within bivalents, and the two variants of a sequence can remain permanently.

Heuberger et al. (2012b) analyzed the ITS-regions (18S - ITS1 - 5.8S - ITS2 - 26S) of several single plants of different cytotypes of V. *officinalis* L. s.l. and compared available valerian sequences from NCBI-GenBank (Benson et al., 2005). In both ITS-regions of some tetra- and octoploid plants, sequence variants were found. The positions and coexistence of the two different ITS-sequences indicate that hybridizations occurred and, hence, an allopolyploid genesis. The results of the genotyping and ITS-region analysis can be understood as an indication of a possible allopolyploid origin, at least for the step from the diploid to the tetraploid level. However, an autopolyploidization followed by a longer, independent evolution of the cytotypes with an introgression by other taxa would also be possible. To clarify the formation of the polyploidy-complex, further investigations are necessary.

1.4 Plant Description

1.4.1 Life Cycle (Ontogeny)

Valeriana officinalis L. s.l. is an herbaceous perennial plant, but it behaves similar to a winter annual during the early life period. The first year is distinguished by predominantly vegetative growth. Subsequently, the plants hibernate with their subterranean plant parts (rhizome) and with their young, vegetative shoot buds. The first generative phase follows after vernalization (Figure 2-A), in which the shoot buds are induced (Figure 3-B, vi). In the following spring, the basal leaves (rosette) appear beginning in February and by internode stretching. The rosettes form a leafy shoot with a terminal inflorescence by internode stretching. After flowering and seed ripening, the inflorescence and basal leaves undergo senescence, followed by the formation of new basal leaves and underground shoot buds

until senescence begins for hibernation. This process enables valerian to renew itself repeatedly for several years.

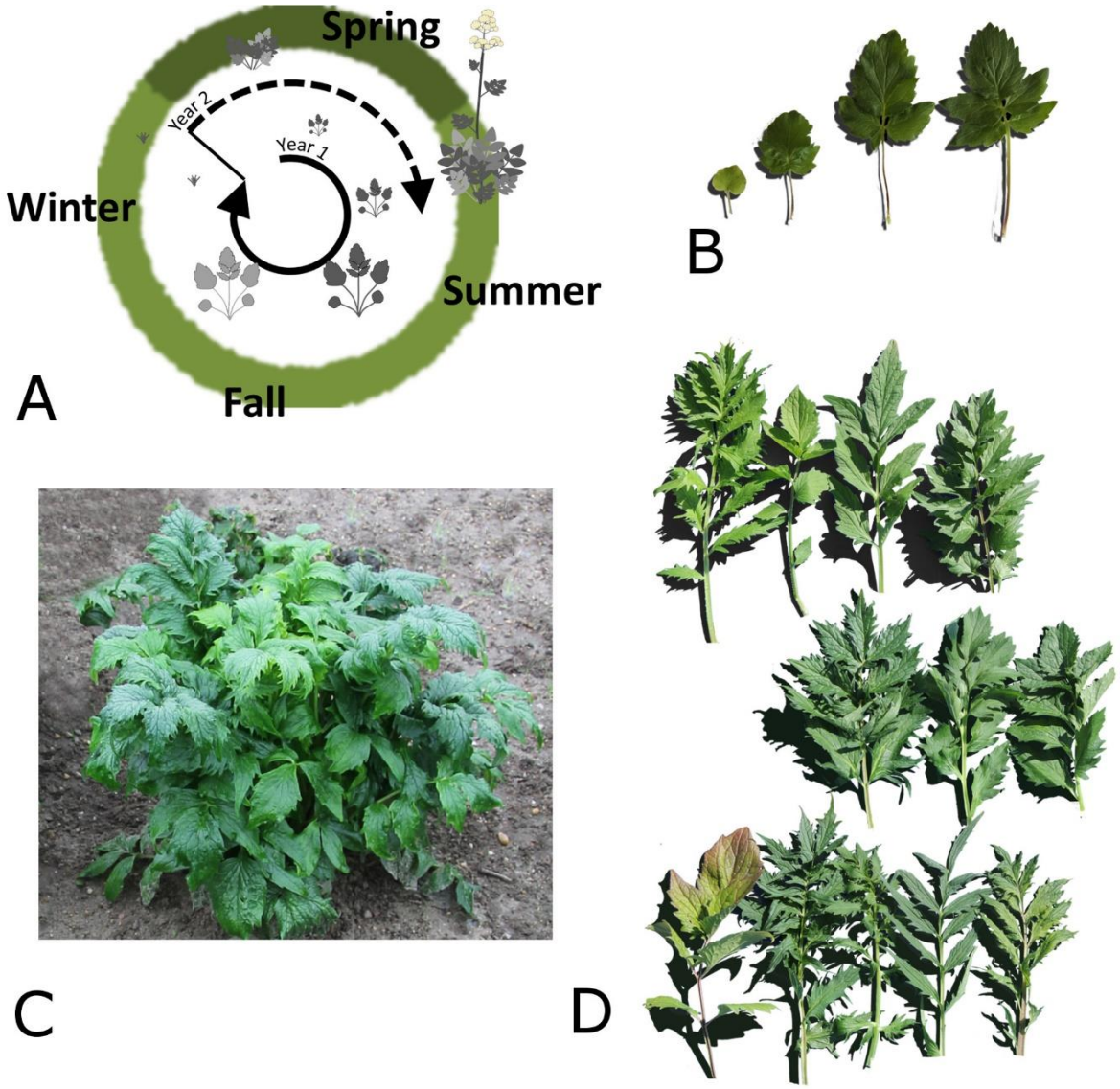

Fig. 2. Morphology during the vegetative phase (first year) of *Valeriana officinalis* L. s.l. A: Stages of development (ontogeny). B: Different leaf shapes during young plant development. C: In the first year, valerian usually forms leaf tufts. D: Different leaf morphologies and leaf textures of tetraploid valerian.

1.4.2 Morphology of Vegetative Plant Parts

The morphology is quite variably, as the complex taxonomy suspected. The plants of *Valerian officinalis* aggregate form imparipinnate leaves, with 7-23, lanceolate to linear and weakly to strongly serrated leaflets (Figure 2-D). From the cotyledon stage to the young plant, the leaf shape changes with each new leaf (Figure 2-B). The leaf color ranges from deep green to light green and can exhibit blue-gray and reddish tints. The leaflet surfaces, petiole and midrib can be more or less hirsute. In the first year, the

plant can reach heights of approximately 30-60 cm (Figure 2-C) (Heeger, 1956; Jäger and Werner, 2002; Gregor et al., 2016).

1.4.3 Morphology of the Inflorescence and Seeds

The *Valeriana officinalis* aggregate forms one to several shoots with terminal paniculate and umbrella-shaped inflorescences and reaches heights of up to 2 m (Figure 3-A). The blooming period lasts from May to August. The flower color is white to pale rose. The corolla consists of five petals and is spurred and asymmetric. Valerian flowers exhibit three stamina and three adnate carpels (Figure 3-B, iii).

The seeds of *Valeriana officinalis* L. s.l. are approximately 3 mm long and form a thousand seed mass between 0.4 and 1.1 g. The seed surface exhibits longitudinal ribs, with a light to dark brown color that is more or less hairy (Heeger, 1956; Bomme, 2001). At the time of seed ripening, the seeds bear a pappus (Figure 3-B, v).

1.4.4 Flower and Pollination Biology

Valerian flowers exhibit each of three stamina and carpels. Dichogamy (protandry) occurs, in which the pollen-covered anthers protrude from the flowers approximately one to two days before stigma opening of the three flaps. The protandry protects the single flower from self-pollination (autogamy), but not from pollination within the inflorescence. Thereby, *Valeriana officinalis* L. s.l. is generally regarded as a cross-pollinated species, which can certainly be suspected by the enormous variability within populations (Heeger, 1956; Heuberger et al., 2012a). The inflorescence exhibits different maturity stages of flowers and seeds concomitantly because of the continuously appearance of new flowers during the blooming period.

Valerian is a perennial herbaceous plant and, during the change from the vegetative to the generative phase, behaves as winter annual plant. Thus, valerian requires vernalization for flowering. The requirement and intensity of vernalization depend on the genetic background, age and cultivation history of the plant material, as well as on the duration and temperature of vernalization. To artificially induce flowering during cultivation, potted plants can be kept in cooling chambers for a minimum of eight weeks at 4 °C with eight hours of light per day (Honermeier et al., 2011; Heuberger et al., 2012c). The number of leaves is a good indicator of the maturity of the plant apex to translate low temperatures into the initiation of generative plant organs. Seedlings should have a minimum number of six to ten leaves. The reaction under cool temperatures of young plants that originated from *in vitro* propagation (cloning) can deviate. Presumably, no flowers are formed because of the imbalance in phytohormones. After flowering and seed ripening, the flowering shoot undergoes senescence, and new vegetative shoot buds are formed. Without further vernalization, the newly formed shoot buds would not undergo flowering.

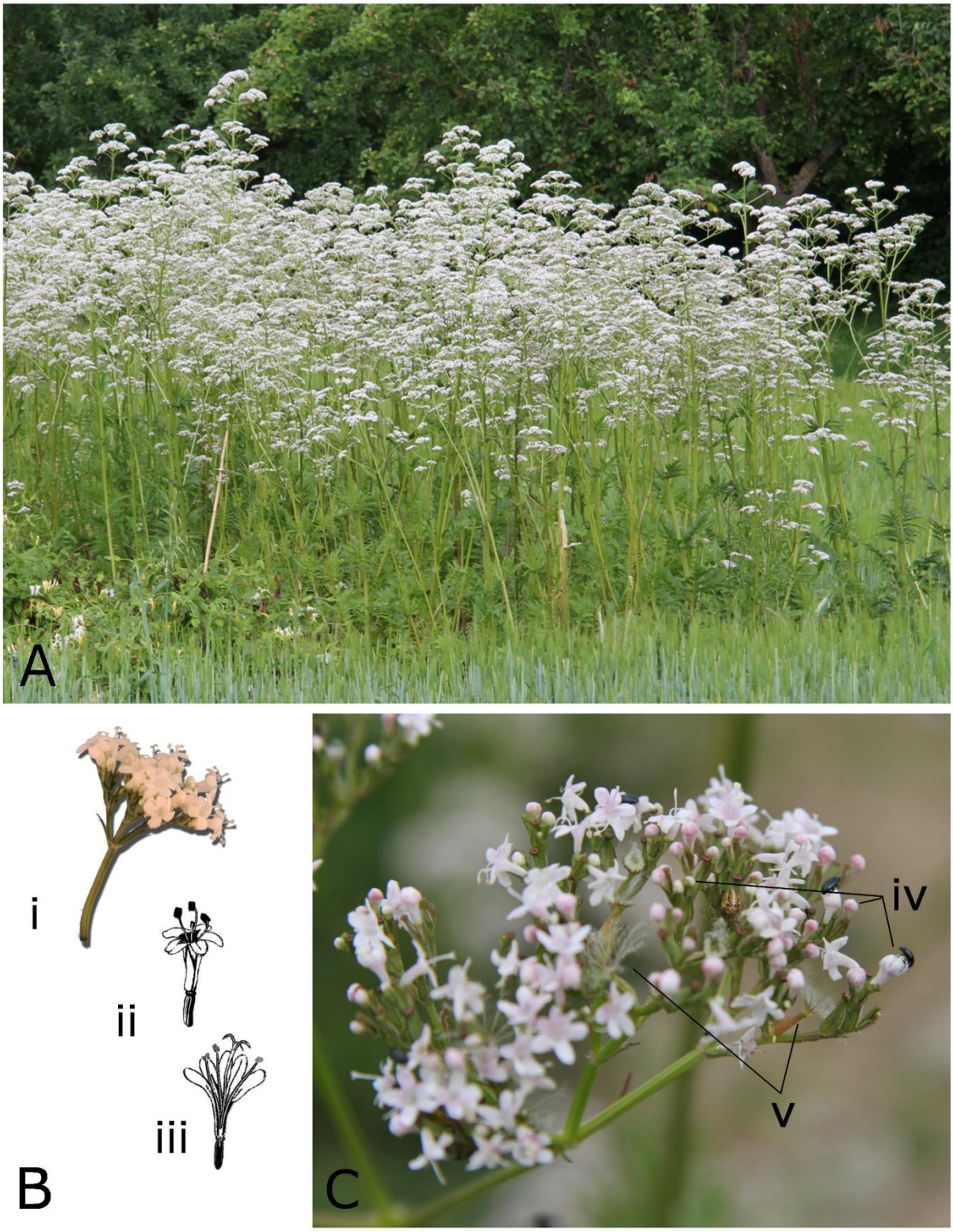

Fig. 3. Development of inflorescences and seeds in the generative phase of *Valeriana officinalis* L. s.l. B: Group of flowering valerian plants. B: Type of inflorescence and flower development stages, i: part of the inflorescence, showing the panicle-like cyme, ii: single protandric flower with mature stamina and immature carpels, iii: single flower with three stigma segments open after pollen maturity, iv-v: inflorescence exhibiting different maturity stages of flowers and seeds simultaneously, iv: different bud stages, v: young seeds with a pappus.

1.4.5 Morphology of the Root System

Valeriana officinalis L. s.l. forms a very fine structured root system. The more or less thick adventitious roots derived from the rhizome, and the thinner lateral roots that are interweaved to form a carpet-like matted network (Figure 4-C). The rhizome is short and cylindrically shaped. The formation of long upper and subterranean runners is often described for wild valerians (especially octoploids) at the habitat origin (e.g., Titz and Titz (1982)), but they are rarely found in cultivated accessions and breeding material (Heuberger et al., 2010). This ergonomically hindering characteristic may have already been eliminated unintentionally in earlier selection works.

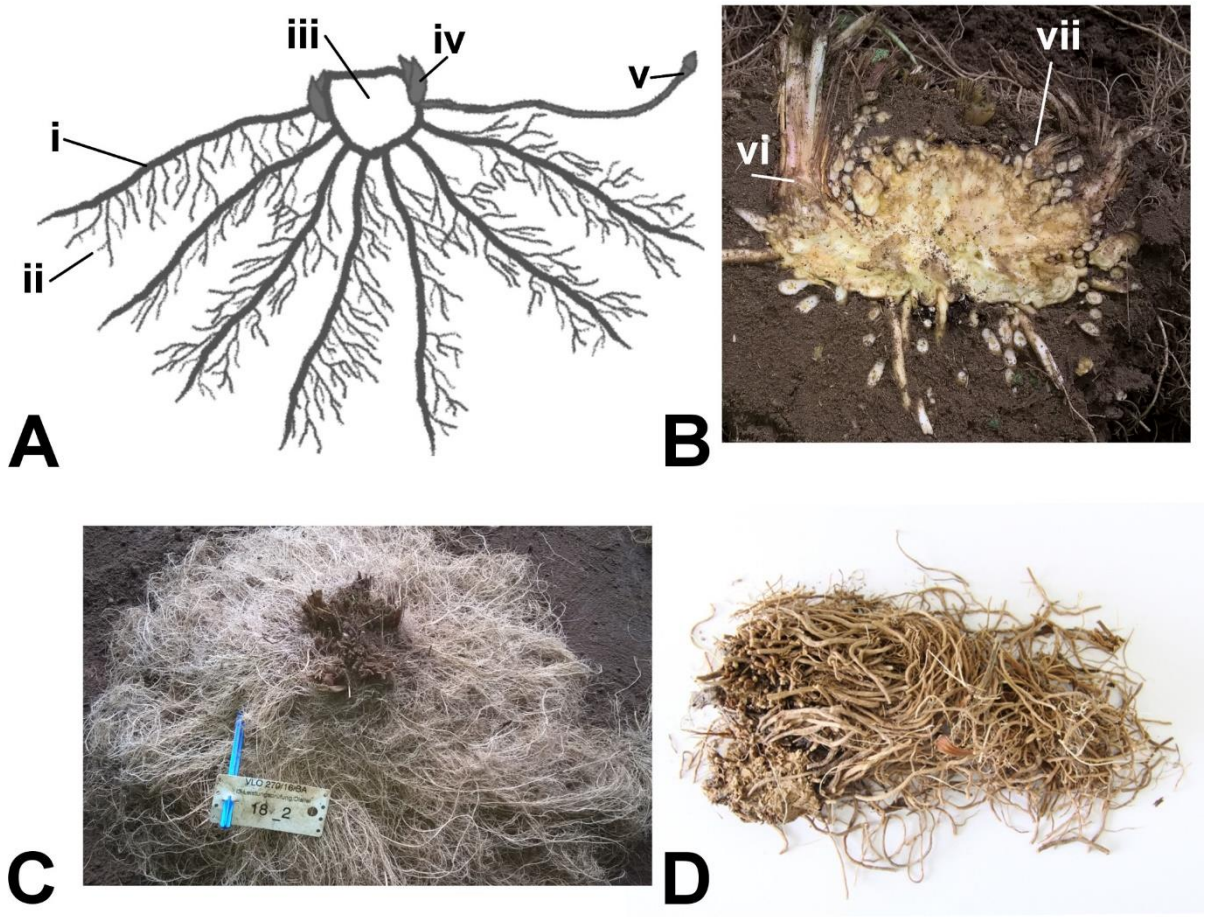

Fig. 4. The root system of *Valeriana officinalis* L. s.l. A: Schematic illustration of the root system, which includes i: adventitious and ii: lateral roots, iii: rhizome, iv: shoots and, if formed, v: runners. B: Vertical cross-section through a rhizome at the vegetative stage before hibernation, vi: base of a new vegetative and unstretched inflorescence shoot, vii: small vegetative bud. C: Topview of a fine structured, carpet-like and matted root system. D: Dried root system, including rhizome and roots.

2 Cultivation and Economical Use

All commercially used valerian raw material is derived from cultivation. The methods of cultivation vary and depend on local conditions and on the individual operating structure.

2.1 Location

Valerian is a robust crop, demonstrating hardy characteristics against spring frost and the ability to be cultivated in rough areas, if sufficient precipitation or irrigation is ensured of approximately 600-700 mm/year. Valerian has no high requirements on the soil, but it grows well in argillaceous peat soils with high humus contents. From a technical perspective, these soils should be avoided because of the difficulties arising from the adhesion of soil particles in the root system during harvest and postharvest processing. For cultivation, porous soils are selected, such as sand or sandy loam, with a low incidence of stones or gravel and low weed infestation, especially of root spreading weeds (Bernath, 1997; Bomme, 2001; ESCOP, 2009).

2.1.2 Propagation Strategies

Valerian can be propagated generatively as well as vegetatively; however, vegetative propagation is used for field production of valerian roots only in special cases. The simplest vegetative propagation is the division of the rhizome into several parts with regenerative buds (Heeger, 1956). Currently, vegetative mass propagation is commonly performed by using plant tissue cultures. Eisenhuth (1966) reported vegetative propagation by runners of the variety 'Merkator', bred in the former German Democratic Republic. *Valeriana wallichii* DC can regenerate from cell suspension cultures and can be propagated well *in vitro* (Mathur et al., 1988; Mathur et al., 1989; Viola and Fritz, 1991; Mathur, 1992). Enciso-Rodríguez (1997) described a method for the micropropagation of *Valeriana edulis* ssp. *procera* by using germinated seeds for the *in vitro* establishment. Seeds from *Valeriana officinalis* L. s.l. were used as starting material for the investigation of rapid *in vitro* shoot regeneration by Tansaz et al. (2014). Penzkofer et al. (2018) described micropropagation by using inflorescences at an early bud stage as starting tissue and side shoots as propagation parts. This method is useful if, for an existing adult plant, a larger number of genetically identical plants is needed.

However, the commercially used method for propagation is the production of seeds. Seed production requires special care and technology because of the continuous ripening and dropping of seeds within the inflorescence. The seeds are harvested as soon as the first seeds turn brown by cutting the flower stalks of the base.

2.1.3 Cultivation

The standard procedure for valerian field cultivation is the planting of young plants in spring (Heuberger et al., 2012a). The young plants are cultivated under protected conditions (greenhouse). The sowing starts in mid-February. The seeds will be placed as a single grain or tuff (3-5 seeds) in multipot plates. At a germination temperature of 20 °C, the duration of the germination phase is approximately one to two weeks. Thereafter, a gradual temperature lowering to 16 °C and a final cooling and ventilated phase for hardening for at least three days is recommended. The well hardened young plants can be planted with a common planter at the end of March to early April. Usual stand densities are 40,000-100,000

plants or tuffs per ha at row distances of 30-50 cm and spacing within rows of 20-30 cm (Heuberger et al., 2012a).

2.1.4 Cultivation Measures

During field cultivation, weed control, irrigation and fertilization are important maintenance acts. Valerian grows naturally on fresh to moist habitats and is classified as a crop with high water requirements. Fertilization is usually carried out before and during cultivation, depending on the availability of nutrients, the fertilizer used, the cultivation system, the developmental stage of the plants and the weather conditions. According to Bomme and Nast (1998), the average nutrient uptakes values are 100 kg N/ha, 48 kg P_2O_5 /ha, 162 K_2O/ha, 21 kg MgO/ha and 90 kg CaO/ha, based on 15 t/ha fresh root mass and 20 t/ha fresh leaf mass. Diseases of valerian are relatively rare and mostly without economic relevance.

2.1.5 Harvest and Postharvest Treatment

The start of the harvesting phase is determined by several parameters. These can be the operational structures and processes, the cultivated varieties and the climatic conditions. The optimum time for root harvest extends from September to Mid-November (Bernath, 1997; Bomme, 2001).

Before root harvesting, the leaves must be cut off as close to the soil surface as possible, which is commonly performed using a mower or beater. The root harvest can be conducted with adapted sugar beet or potato harvesters (Bomme, 2001; Neumaier, 2017) or harvesters for tree nurseries.

Due to the very fine structured root system, the freshly harvested root system can contain up to 75 % soil mass (Heuberger et al., 2012a). Following the harvest, several cleaning steps are needed to remove the soil and reduce the content of HCl insoluble ash to the required maximum content of 5 % and the percentage of other impurities to 2 % (Ph.Eur.9.1, 2017). Depending on the individual procedure, the root stocks are comminuted, freed from bulk soil and stones and washed with water. Different combinations of choppers, rippers and sieves are used. The washing is carried out often with drum washing machines using high water rates.

The washed roots are dried at 40-45 °C with high air volumes in flat dryers or drying hurdles, performed as intermittent drying that allows the water in the rhizomes to migrate to the tissue surface. Belt dryers are less suitable because of the long drying time and poor flowing properties of the root material. The maximum allowable residual moisture content of 12 % is reached after 20-40 hours of net drying time (Heuberger et al., 2012a; Ph.Eur.9.1, 2017; USP41, 2017).

2.2 Valuable/Undesirable Plant Secondary Compounds

The valerian root drug contains a large number of secondary compounds, which have been isolated and characterized from the root and the rhizome (Trauner, 2009; Wichtl, 2009): sesquiterpenes (Stoll and Seebeck, 1956), iridoids (Valepotriate) (Thies and Funke, 1966), lignans (Bodesheim and Hölzl, 1997; Schumacher et al., 2002), flavonoids (Fernández et al., 2004; Marder et al., 2003), alkaloids (Torsell and Wahlberg, 1966; Gross et al., 1971), amino acids, phenolic carboxylic acids, sterols, carbohydrates

and others. A detailed compilation and overview of the diverse substance groups and ingredients is provided by Heuberger et al. (2012a). The characteristic smell of valerian develops during the drying process; it is mainly caused by the liberation of isovaleric acid (Sticher et al., 2015).

The components described in this work are contained in larger quantities in the root drug and in essential oil, respectively, or are important for the medical application and the determination of the root drug quality. The contents and composition can be quite diverse and are influenced by the plant genetic properties, the origin of the plant material and the method of extraction (Bos, 1997; Houghton, 1997).

The valerian root drug contains 0.3 to 1.0 %, and in some cases over 2.0 %, essential oil (Houghton, 1997; Wichtl, 2009; Heuberger et al., 2012a). The essential oil content is determined by water distillation (Ph.Eur.9.1, 2017; USP41, 2017). The quality standards for the minimum essential oil content are defined by the European Pharmacopoeia as 4 ml essential oil/kg (equates to 0.4 %) for the uncut root (*Valerianae radix*) and 3 ml essential oil/kg (equates to 0.3 %) for the cut root (*Valerianae radix minutata*) (Ph.Eur.9.1, 2017). The essential oil is composed of a mixture of monoterpenes and sesquiterpenes; until now, approximately 150 components have been identified. The most frequent major constituents of essential oil of *Valeriana officinalis* L. are the monoterpenes borneol and its esters bornyl acetate and bornyl isovalerate. Further monoterpenes of the essential oil are camphor, camphene, 1,8-cineole, α-pinene and myrcene (Stoll et al., 1957; Reichling et al., 1994; Houghton, 1997; Bos, 1997). The most frequent sesquiterpenes are valerenal, valerenol, valerenyl acetate, valerenyl isovalerate, kessan, kessanyl acetate, α-kessyl acetate, faurinone, patchouli alcohol, α-curcumene, β-bisabolene and others (Houghton, 1997; Reichling et al., 1994; Bos, 1997). Valerenic acid, hydroxyvalerenic acid, acetoxyvalerenic acid and 3β,4β-epoxyvalerenic acid are cyclopentane-sesquiterpenes and form a subgroup of the sesquiterpenes (Stoll and Seebeck, 1956; Reichling et al., 1994; Houghton, 1997; Dharmaratne et al., 2002).

These low-volatile sesquiterpene acids are typical for *Valeriana officinalis* L. s.l. and can be used as marker substances in the quality control of valerian preparations (Hänsel and Schulz, 1982; Navarette et al., 2006; Ph.Eur.9.1, 2017). The quality standards for the minimum content of sesquiterpene acids (valerenic acid and acetoxyvalerenic acid), calculated as valerenic acids, are defined by the current European Pharmacopoeia as 0.17 % sesquiterpene acids for the uncut root (*Valerianae radix*) and 0.10 % sesquiterpene acids for the cut roots (*Valerianae radix minutata*) (Ph.Eur.9.1, 2017).

An additional group of compounds that appear in valerian are the valepotriates. These bicyclic monoterpenes are instable compounds and will be decomposed quickly under acidic or alkaline conditions or by the influence of heat (Bos, 1997; Wichtl, 2009). Extracts from the roots of *Valeriana officinalis* L. contain up to 2 % of a mixture of valepotriates. Currently, understanding of the medically active substances has changed through intensive research. Valepotriates act as mutagenic agents after metabolic activation. Thus, the importance of valepotriate-rich preparations has been lost (Sticher et al., 2015).

The effectiveness has thus far attributed to different groups of elements. Sticher et al. (2015) classified the lipophilic group (including, e.g., the sesquiterpenes, sesquiterpene acids and borneol) and hydrophilic group (including, e.g., lignans and flavonoids) as active substance classes. The mechanism of action is probably caused by the linkage of extractives or single ingredients to adenosine receptors, subtypes of the serotonin receptors and the GABA receptor complex, respectively (Sticher et al., 2015). The latest investigations have revealed that valerenic acids stimulate subtypes of $GABA_A$ receptors, initiating a soporific effect (Khom et al., 2007; Benke et al., 2009; Becker et al., 2014). Murphy et al. (2010) identified valerenic acid as the substance with the highest anxiolytic effect in valerian. Acetoxyvalerenic acid also binds to GABA receptors, but without an anxiolytic effect, thus inhibiting the potential of valerenic acid (Felgentreff et al., 2012).

2.3 Economic Valuation/Parameters

Valeriana officinalis L. s.l. is a classic medicinal plant in western medicine. The dried root system is used as a raw material mainly for extraction, in smaller volumes for essential oil distillation and for herbal tea production. In Germany, the quantified demand for dried valerian roots is approximately 1,000 tons and forms a market size of approximately 4 Mio. € (FNR, 2013; FNR, 2014).

The production of dried valerian roots is highly labor and cost-intensive due to the necessity for crop establishment by planting as well as the time and energy-consuming harvesting and drying of the roots and rhizomes (Heuberger et al., 2012a). Approximately 60 % of the production costs are caused by drying, processing and personnel, and 30 % must be calculated for the planting material (young plants) (Bomme et al., 2002; Heuberger, 2014).

3 Applied Breeding Methods and Techniques

In comparison to other agricultural crops, the breeding of valerian – as well as of many other medicinal and spice plants – is at an early stage of development (Hoppe, 2009). Innovations in breeding and cultivation techniques are closely interrelated and can influence each other.

Bernath (1997) summarized the development of valerian breeding into four periods, reflecting the chronological development. In the first period, dated back to the early 1930s, mass-selection and simple selective breeding was performed in native wild populations. Attention was focused on morphological and production characteristics, especially root productivity. The only chemical character that was taken into consideration was the essential oil. The second period (1950s) is characterized as the time where mass-selection was expanded and systematic analyses of populations achieved (Eisenhuth, 1956). The aim of the third period (as of the 1970s) was the stabilization of root yield and quality, mainly of the ingredient contents and composition. The persistent high variability of the plant material has been a risk for meeting the demand of the pharmaceutical industry for constant raw material. The late 1980s, the fourth period, is characterized by the application of new genetic knowledge and the use of traditional and new methods of plant breeding.

In contrast to other medicinal plants, an unusually large number of valerian varieties have been bred. Many of them are local varieties and are not registered in a variety index. Many are also no longer available in the seed trade (Heuberger et al., 2012a). Concerning the development of these varieties, no or solely little information has been documented. Penzkofer and Heuberger (2019) described valerian breeding based on their own findings and experiences and provided references where possible.

3.1 Breeding Techniques

The creation of new variability and selection are the basic process steps that are used in any type of variety development. For this purpose, different techniques and methods have been developed. In this context, the technique is to be understood as a treatment or procedure used in breeding, and the method is the application of different techniques during the breeding process. A universal scheme or procedure does not exist for valerian. However, a multitude and often also a mixture of different breeding techniques and methods are applicable, depending on the breeding target and the facilities of the breeder.

3.1.1 Creation of New Variability

The fundamental step during breeding is the creation of new variability. Pollination control is the one most often used, as well as the simplest technique, and it can be performed in two directions. Either the combination of geno- or phenotypes or the exclusion of unwanted geno- or phenotypes. The use and manipulation of ploidy levels is mostly more complex and is predominantly used in the primary stages of breeding or in research.

Control of Pollen Transfer

The *Valeriana officinalis* L. s.l.-complex exhibits a certain variability such that crossing within the complex is sufficient for the creation of new variability. An essential aspect of breeding is the control of pollination (Acquaah, 2007). The targets of these pollination control techniques are ideally to maximize the proportion of hybrid or inbred seeds among the offspring.

Pollen transfer between different geno- and phenotypes leads to hybridization and can be controlled or maximized by the application of mechanical/technical or chemical treatments, or by using the genetic characteristics of the parent plants, which influence the sexual biology (Brown and Caligari, 2008). Mechanical/technical pollination control is often based on the removal of anthers from the bisexual flowers of valerian. Kempf (1986) elaborated some basic information about emasculation (castration) techniques. Manual emasculation is in principle possible, but in most cases, it is too time consuming and expensive for the low seed yield, which can be generated (Acquaah, 2007). Therefore, it is mainly used for scientific investigations (Penzkofer et al., 2014a; Heuberger and Penzkofer, 2017).

To generate a higher castration rate, the hot water emasculation technique was developed in grain and rice breeding, and it was successfully applied in further crops (Mukasa et al., 2007; Tong and Yoshida, 2008; Otsuka et al., 2010; Hussain et al., 2012; Stetter et al., 2016). Valerian shows no or only a slight difference in temperature sensitivity between male and female flower parts, so hot water emasculation could not be successfully initiated (Kempf, 1986).

Male sterility can support cross-pollination. Shugaeva (1979) described valerian inflorescences with completely reduced anthers and total pollen sterility. Furthermore, plants containing normally developed androgynous flowers as well as flowers with male sterility simultaneously were also described. The ratio of the two types of flowers varied from plant to plant and changed during the process of flowering (Shugaeva, 1979). To use male sterility during breeding and in seed production, it is necessary that the inducing principle for male sterility (genomic or cytoplasmic) is known and is not manipulated by noncontrollable external influences. Furthermore, maintenance of the sterility system must be possible.

The prohibition or restriction of pollen transfer between different geno- and phenotypes is the second aspect of pollination control. Isolation means that the transfer of pollen between different plants is prevented. The classic proceeding is the isolation of one valerian plant or inflorescence. This is done predominantly for developing homogeneous descendants (inbred lines) (Vömel and Hölzl, 1979; Konon and Novikova, 1981). Many single-plant isolations can be performed in a greenhouse concurrently (Figure 5).

Depending on the purpose of isolation, as well as for more than one plant and for plants with different genetic configurations, isolation techniques can be applied. The isolation of two inflorescences of different plants promotes the intended crossing since a high outcrossing rate can be assumed, as reported by Penzkofer et al. (2018). If more plants are to be crossed together, the use of isolation cabins allows improved handling. The isolation cabins are covered with gauze frames and can be placed in greenhouses or, as shown in Figure 6, outdoors.

Fig. 5. Inflorescences of valerian individually isolated with glassine bags in a greenhouse.

Fig. 6. Blooming valerian in an isolation cabin in the field.

Manipulation of the Ploidy Level

The naturally present different cytotypes in valerian provides the possibility of creating new variability and new plant traits. Penzkofer et al. (2014a) described reciprocal crosses between di-, tetra- and octoploid origins of valerian. The cross-pollination was carried out by emasculation (castration) and

manual pollen transfer. The inflorescences were protected against foreign pollen by isolation. The ploidy level of the descendants was determined using flow cytometry and microscopic chromosome counting.

Not all descendants showed the cytotype, which would be expected due to the ploidy of the crossing parents. The crossing of an octoploid mother and diploid father led to a triploid descendant, although a pentaploid was expected. A similar phenomenon was observed by the crossing of tetra- and diploids, what resulted in a tetraploid (a triploid was expected). Varying ploidy levels within the mother plants could be excluded. The observed results may be caused by distorting effects during meiosis or incomplete emasculation, enabling self-pollination.

Reduction and doubling (polyploidization) of the chromosome sets, also called the double-haploid-technique, are biotechnological procedures that are also applied in breeding of medicinal plants (Ferrie, 2009). A reduction to a monoploid (usually a haploid) with subsequent doubling leads to homozygous plant material. This technique to create homozygous plant material provides an alternative to continuous inbreeding and is often upstream to hybrid breeding. Due to the unknown origin and unknown types of polyploidy of tetraploid valerians, two reduction steps and two doubling steps would be necessary each to ensure full homozygosity. Several attempts to develop viable haploid valerian plant material were unsuccessful (Bal and Touraev, 2009; Nietsch, 2010; Göttl, 2011).

After reduction, a doubling of the chromosome sets is usually performed. Doubling of the chromosome sets in valerian is possible by applying colchicine (Heuberger et al., 2012c). Thus far, these efforts have been unsuccessful because the technique was not able to ensure that all cells, which were able to divide, were in fact being treated. It was not able to detect whether the generated plants contained two complete sets of chromosomes. Reduction, doubling and crossing of different cytotypes can be used for the development of a polyploid series (e.g., from 1x to 8x). Such a polyploid series can provide information on whether the performance of valerian can be improved with increasing numbers of chromosomes. Because valerian accessions with different ploidy levels also differ in their genetic compositions (Heuberger et al., 2012b), a polyploid series of valerian must be based on the same haploid (monoploid) plant. There are no reports of the successful development of a polyploid series of valerian.

3.1.2 Selection

Selection presents the most important tool during breeding. The response to selection usually depends on the selection intensity, the variability of the parental population and the heritability (genotype-environment-interaction).

The selection intensity depends on the possibilities of the breeding work and the existing (amount) plant material. A high genetic variability exists among populations and can also be observed within most valerian populations. The finding of plant material with wanted characteristics should not be problematic.

A challenge during each selection process is to recognize the appropriate plant material as soon as possible and to receive the plant material nondestructively and without loss. If the performance of valerian should be determined in the usual field cultivation system, the root system, being the most

important plant part, is harvested in autumn of the first cultivation year. Visual evaluation of the root system, yield measurements and chemical analyses ensue. Hence, just one selection step can be conducted in one year, and the entire selection process will inevitably be stretched.

In general, selection can be based on genotypic and/or phenotypic properties. However, genome-based selection does not yet play a role in valerian breeding, and whether such techniques are used is unknown.

The possibilities of phenotypical selection in valerian range from the morphological characteristics of the root system (e.g., root structure), the aboveground plant parts (texture and habitus) and the inflorescence (e.g., flower time and range). The most important agro-economic parameters are the root and seed yield.

Analytical selection applies predominantly to the secondary compounds indicated in the pharmacopoeias (Ph.Eur.9.1, 2017; USP41, 2017), in which the analytical methods and procedures are described (e.g., water distillation, thin-layer chromatography, liquid chromatography (HPLC)). Analysis of the secondary compounds can be performed for single plants, or for a group of plants based on a mixed sample.

The contents of secondary compounds are very variable traits, which are influenced by the genetic constitution as well as by environmental conditions, the stage of plant development (ontogenesis) and the processing of the plant organ up to analysis (Hörner 1989; Noller, 1989; Bernath, 1997; Bos et al., 1998). The different secondary compounds of valerian do not behave in similar ways. According to Hörner (1989) and Bos et al. (1998), the content of essential oil increases up to the end of the vegetative phase and decreases with the development of the generative plant parts. The content of valerenic acid reaches the highest values during or just before of the flowering period (Noller, 1989; Bos et al., 1998). Bernath (1997) discusses the influence of growth factors (light, temperature, water requirement, soil conditions, nutrition) on secondary compound production and describes quite different results.

3.2 Breeding Methods

The above-described breeding techniques are expedients for applied breeding work and can used in diverse ways. In the following section, two methods are presented, which utilize the described techniques and are based on self- or cross-pollination strategies.

Method Using the Self-pollination Strategy (Inbreeding)

Inbreeding means recurrent pollination with self-pollen (self-pollination). The main aim of inbreeding is to create greater homozygosity in the plant material. The fully inbred plant material no longer shows allele variation, and all or nearly all loci are homozygous. The gene combinations are fixed and can be identically reproduced.

As early as the first inbred generation, inbreeding depression occurs in valerian and is enforced with increasing inbreeding levels. The inbreeding effect on vitality-indicating characteristics, such as the time until full ground coverage is reached, the vigor (plant height) or the seed yield, illustrates that

further inbred steps over the third inbred generation are very difficult to execute and often lead to inviable plant material.

The majority of cultivated valerian are tetraploid. In comparison to diploids, it is more difficult to identify homozygous plants and to determine the frequency of dominant alleles due to five possible allele combinations, from nulliplex (aaaa) to simplex (Aaaa) and to quadruplex (AAAA) (Schmalz, 1989; Stoskopf et al., 1993). In addition, a longer time is needed to reach full homozygosity using classic inbreeding steps. In diploids, the theoretical portion of 97 % of homozygous dominant alleles would be reached in the sixth inbred generation. In contrast, in the case of tetraploids such as valerian, such a level of homozygosity is statistically reached after the 21st generation starting from a duplex genotype or after the 19th generation starting from a triplex genotype. Considering the high inbreeding depression, as shown for the third inbred generation, reaching full homozygosity by classic inbreeding is not possible for valerian. Despite all these challenges, different generations of inbred lines of valerian can be developed (Konon and Novikova, 1981; Kempf, 1986; Penzkofer and Heuberger, 2018).

Method Using Cross-pollination Strategies (Population-breeding, Cross-breeding, Hybrids and Synthetics)

Due to the high tendency toward natural cross-pollination (xenogamy), methods of population breeding with open pollination are applied for valerian. The simplest method for valerian seems to be mass-selection followed by seed production by open pollination. In Germany, this process has been documented since the end of the 18th century (Heeger, 1942).

The selection of individuals is still an appropriate method for starting new breeding programs of valerian (Heuberger et al., 2012c). The selection can be done before or after flowering, with different emphases of the criteria. The criteria, which are assessable prior to the flowering time, are mostly important for growers, whereas after- or during-flowering criteria are important for seed producers and the breeder. A selection before flowering is useful to reduce the genotypic frequency of recessive-negative alleles (characteristics). The pollen of such individuals is no longer available for pollination. A two-step selection is advised, by which individuals are selected before flowering according to the agronomic and root quality criteria, and subsequently during or after flowering, the performance of these selected individuals can be considered in the generative phase. Targeted cross-breeding is used for valerian breeding to combine different characteristics, which are missing in one parent each (Heuberger et al., 2012c). Ideally, each individual combining partner as well as the resulting combination (offspring) show good performance. For valerian, comprehensive hybrid performance and the combination of ability tests have not yet been executed. Penzkofer and Heuberger (2018) investigated the offspring (F_1) of four crossed inbred lines (I_3). The examined progenies showed different values for negative and positive mid parent heterosis (MPH), both between the inbred lines and between the characteristics. In summary, crossings between different inbred lines exhibit a higher MPH than crossings of the same inbred lines.

The combining ability of the inbred lines (general combining ability, GCA) or of the single combinations, where single plants acted as combining partners (specific combining ability, SCA), are applied as benchmarks for the successful development of a hybrid variety. The insignificant SCA and GCA values for the "thickness of adventitious roots"-characteristic are conspicuous because thick roots are the main breeding aim of the breeding project. With the consequent selection geared toward thick-rooted inbred lines, the root thickness in the F_1-offsprings may not be further increased by crossing, or the root thickness rating of the F_1-offsprings may be too imprecise, because of the resemblance of the offspring.

3.4 Breeding Targets

The breeding targets for valerian have changed several times in the past years. The breeding targets are always to be considered in connection with the state of the existing breeding and cultivation techniques and the re-evaluation of the secondary compounds.

In contrast to other medicinal crops, the cultivation of valerian is rather uncomplicated, predominantly well investigated and well established. Breeding works for plant characteristics relevant during early cultivation and early processing steps (seed production, crop establishment and field cultivation) were performed, if at all, solely as secondary aspects.

More attention is currently given to the harvest and postharvest treatments (root harvest, cleaning and drying) because they are cost-intensive processes and are mostly likely to incur quality losses. The very finely structured and very strong interweaved carpet-like matted network of the root system is deep-seated in the ground and must be harvested and processed with considerable expenditures. A high mechanical strain on the root system, in which roots can break, can lead to a decrease in root yield. Furthermore, adherent soil must be washed away extensively, resulting in losses of secondary compounds. Valerian varieties with thicker and less branched root systems, in combination with a high root yield and high content of secondary compounds, would counteract this loses. In addition, varieties with smaller rhizomes would be beneficial for faster and less energy-consuming drying.

In 2009, a valerian breeding project was started with the aim of developing one or more valerian varieties with thicker and less branched root systems (Heuberger et al., 2012c; Heuberger and Penzkofer, 2017; Penzkofer and Heuberger, 2018). The present thesis is strongly related to this current breeding project and should be considered in its context.

2. The Thesis Objectives

The thesis objectives are related to a current breeding project, which central breeding aim is to develop new valerian varieties with a coarser root system (Heuberger et al., 2012c; Heuberger and Penzkofer, 2017; Penzkofer and Heuberger, 2018). The new varieties must comply in minimum with the quality regulations of the European Pharmacopoeia (Ph.Eur.9.1, 2017). Due to the characteristics, root morphology and contents of secondary compounds, it is particularly important to understand the relationship between them and to examine them in the current breeding material. Undocumented information of growers and of the initial breeding material (Figure 7) led to the assumption that thicker roots contain less ingredients and the essential oil is mainly localized in the lateral roots

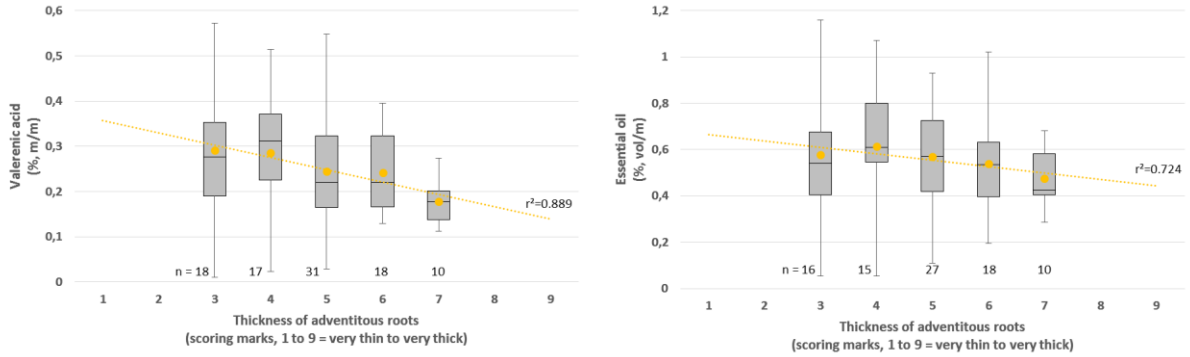

Fig. 7. Contents of valerenic acid (sum of valerenic acid and acetoxyvalerenic acid, left) and essential oil (right) in selected single plants (elites), with different thicknesses (root diameters) of adventitious roots. The grey box describe the first and third quartile the horizontal line in the grey box the median and the vertical lines the minimum and maximum contents. The interrupted yellow line represents the regression line and r^2 the coefficient of determination of the mean values (yellow dots). Data source: (Heuberger et al., 2012c)

Therefore, three research questions were the basis of the first part of this thesis:

- Is there a (negative) relationship between the ingredient content and root diameter?
- In which parts of the root system are the secondary compounds localized?
- How do the secondary compound localization and distribution influence the production of the dried root drugs and breeding?

Because breeding aims at hybrid or synthetic cultivars and lacks a usable hybrid mechanism, the knowledge about the expected proportion of allogamy or autogamy under open pollination conditions, is important for developing the breeding strategies. In the end, the variety seeds should be produced by open pollination and just a minimum proportion of inbred seeds will be accepted. Therefore, the second part of this thesis aimed at answering the following questions:

- What is the proportion of outcrossing under conditions of open pollination in the breeding material?
- What is the probability to develop hybrid- or synthetic-like valerian varieties?

3. Contents of essential oil, valerenic acids and extractives in different parts of the rootstock of medicinal valerian (*Valeriana officinalis* L. s.l.)

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Contribution of other authors:	Elke Ziegler performed 50 % of experiments in her bachelor thesis Heidi Heuberger contributed to experimental design, data analysis and writing the paper

3.1 Manuscript

Abstract

In 2008, a breeding project was started with the aim to develop one or several varieties of *Valeriana officinalis* s.l. L. with a coarse root system and reliable ingredient contents according to the pharmacopoeia. It was initially assumed that a negative relationship between the adventitious root thickness and the ingredient contents exist.

Therefore, the clones of four genotypes, differing in root fineness and ingredient content, were cultivated. The rootstocks were carefully harvested by hand in autumn and the rootstock components, rhizomes and roots, were separated. The roots were assigned to four diameter groups and the lateral roots were separated from the adventitious roots after drying. The analyses of the valerenic acids, essential oil and formerly listed extractives were conducted according to the current European Pharmacopoeia.

In general for the investigated genotypes, the highest contents of valerenic acids and essential oil are located in the adventitious roots, followed by the lateral roots and the rhizomes. In contrast to the other ingredients, the extractives content of the rhizomes are comparatively high. Differences in the investigated ingredient contents and the different root thicknesses are not detectable.

The investigation indicates how important careful harvesting and preparation of the rootstock are to conserve the existing ingredients. In addition, the different ingredient contents of the adventitious and lateral roots are not expected to decrease ingredient contents of the root drug when lateral roots and thin adventitious roots are lost during root cleaning and preparation. The study also shows that despite initial assumptions, thicker roots did not result in a decline of ingredient contents.

Keywords: Valeriana officinalis L., valerenic acids, essential oil, adventitious root, lateral root, rhizome

1. Introduction

The medically used valerian

The valerian used in Europe for medical purposes, *Valeriana officinalis* L. s.l., occurs mainly in the temperate zone of the northern hemisphere on fresh to moist habitats (e.g. moist deciduous forests, ditches and banks). It is a diverse family complex, indicated by the suffix (sensulato). Valerian is an herbaceous perennial plant with a very variable aerial habit. The leaves are imparipinnate, the leaflets weakly to strongly serrated. Inflorescences develop in the second year after a vernalization (Heuberger et al., 2012b). For medicinal purposes, the entire root system including rhizomes is used (Ph.Eur.7.0, 2011).

Valerians have been used in phytotherapy since antiquity, for relieving many symptoms of illness (Mayer, 2003). Nowadays, preparations with valerian root are used against restlessness and sleep disturbances (ESCOP, 2003; ESCOP, 2009). Furthermore, valerian improves the mental state during the day and has an antispasmodic and muscle relaxant effect (Schilcher et al., 2010).

The understanding of the medically active substances has changed through intensive research. It is now known that the valerenic acids from the cyclopentan-sesquiterpenes are the medically most relevant ingredient group. The importance of valepotriates has been lost due to the suspicion of a toxic effect (Hölzl, 1986). The current European Pharmacopoeia (Ph.Eur.7.0, 2011) defines a minimum content of 0.17% valerenic acids and 4 ml · kg^{-1} essential oil for the root drug (*Valerianae radix*).

The demand for dried valerian roots in Germany (2011) is quantified at approximately 1000 tons (FNR, 2014). The production of valerian root drug is highly labor and cost intensive, due to the necessity of usually one hoeing by hand as well as the time and energy consuming harvest and drying of the roots. These factors contribute to the small amount of German valerian cultivation (Heuberger et al., 2012b). Valerian is cultivated in Germany to less than 50 ha; however, a growth potential for valerian in Germany is anticipated (FNR, 2014).

Importance of the localization of the ingredients in the root geometry

There is much speculation as to where the valerenic acids and the essential oil are localized within the valerian rootstock. Several descriptions about the localization of essential oil can be found in literature (Tschirch and Oesterle, 1900; Fridvalszky, 1957; Holzner-Lendbrandl, 1963; Kutschera, 1992). The information mostly describes the distribution in the cross-sectional of the roots. A spatial localization or a comparison between different parts of the rootstock has been described by (Eisenhuth, 1956) and has been presented by (Argyropoulos et al., 2013). It is indicating that all rootstock components contain essential oil.

There are no reports about the distribution of valerenic acids. One reason for this is due to the fact that the essential oil was rated for a long time as the most important ingredient. Stoll and Seebeck (1957) were probably the first, to describe valerenic acids in the roots and rhizomes of valerian. (Hänsel and Schulz, 1982) identified valerenic acids as a lead compound. Furthermore, it is not possible to find the valerenic acids by histological studies based on microscopic methods.

During the harvesting, cleaning and drying processes, parts of the adventitious and lateral roots (Fig. 1) are lost, resulting in drug yield losses. Thus, it is not clear to what extend this is also connected to losses of ingredients. This could result in the quality of the valerian drug being reduced. Schunk (1962) reported that the finest roots (fibrous roots, < 1 mm) are among the fractions of the valerian drug, which are characterized by very high contents of essential oil. Therefore, their loss should be avoided as much as possible during harvest.

The localization of the ingredients can be also very important for the breeding work. In 2008, work began with the breeding of new varieties of valerian (Heuberger et al., 2012a). The breeding goal was to create varieties with coarser rootstocks, combined with good ingredient contents. This was intended to reduce the losses during harvest and processing, as well as to simplify the cleaning of the roots. Questions arose as to whether a negative relationship between the root thickness and the amount of ingredients exists. If this is true, the breeding concept would need to be reconsidered.

This leads to two questions that should be answered in this work. First, in which rootstock components are located the ingredients valerenic acids, essential oil and extractives. Second, contain thicker roots less ingredients. In addition, differences between thin and thick rooted valerian populations should be estimated.

2. Materials and Methods

2.1. Plant material

The different rootstock components, usually formed by valerian are illustrated in Fig. 1. In this study, they are named as rhizome, adventitious root and lateral root. In addition, there are also runners and shoots; however, the runners were not formed by the plant material in this study.

In 2010, four plants were selected from the varieties 'Anton' and 'Lubelski', because of their differing root morphology and pharmaceutically relevant ingredient components (Table 1 and Table 2). The data represent single values of the four selected elites (individual plants). Two of the elites, 710177 and 710209, were characterized by a highly branched and felted rootstock and predominantly thin adventitious roots. In the following, these two elites and their derived clones are named as thin rooted. The rootstock of the elites 710237 and 710323 were chunkier, less felted and had thicker adventitious roots. These two elites are referred to as thick rooted.

For the chemical analyses, a minimum amount of dried roots is needed. Therefore, the four elites were cloned (see section 2.2. Propagation and cultivation) in order to obtain sufficient genetically identical material. In the following the genotype is referred with the elite number. To clarify, if the clones are addressed, the elite number is appended the extension "c".

2.2. Propagation and cultivation

Division is a traditional method for cloning valerian; however, due to the low propagation rates, the quantities required for the investigation could not be achieved with this method during this time. Therefore, a sterile micropropagation, using inflorescences at an early bud stage of vital donor plants was used. In the beginning, young and small inflorescences (0.5-1.5 cm) were removed, sterilized in 3% sodium hypochlorite for 20 minutes and dissected. The applied culture media were all based on the compilation of (Murashige and Skoog, 1962). The establishing media received an addition of 0.5 mg/l 6-benzylaminopurin (BAP) and the growing and rooting media received 5 mg/l BAP. The climate conditions in the growing room were regulated at a temperature of 22-24 °C and at a lighting time of 12 hours per day. With this method, the four elites were propagated by side shoot cuttings in several subcultures. After the cultivation in lab and before transplanting to the field, the clones were transferred for the acclimatization to a greenhouse (17th Oct. 2011).

The clones were cultivated at the experimental station Baumannshof, Forstwiesen of the Bavarian State Research Center for Agriculture. It is located 362 m above sea level and is characterized by a humic sandy soil with a pH of 5.0 to 6.7 and anorganic matter content of 1.5 to 2.2%. The field was prepared by ploughing in autumn 2011 and grubbing in spring 2012. The cultivation on the field started

on 28th March 2012 by planting 30 plants for each genotype. The distance between the rows was 42 cm; the distance between the single plants were 30 cm. Before planting, the available plant nutrients in soil were determined. At the site, 50 kg P_2O_5/ha and 160 kg K_2O/ha were deployed as superphosphate and Korn-Kali® with 40% potassium oxide and 6% magnesium oxide. Nitrogen fertilization was carried out in two 40 kg N/ha applications as calcium ammonium nitrate and took place on 18th April and 23rd May 2012.

In 2012, the total annual precipitation was 715 mm; the mean annual temperature was 9.2 °C. In dry conditions, irrigation was conducted when needed. In July and August, four units (each unit contained 20 mm) were released by overhead irrigation. The monthly precipitation and temperatures in 2012 at the experimental area are shown in Fig. 2.

The weed growth was controlled by milling twice and several hand weeding cycles.

2.3. Harvesting

After a growing period of about seven months, the roots were harvested within ten days starting on 25th Oct. 2012 (Fig. 2). The rootstocks were harvested manually in order to salvage the complete root mass. Around the rootstock of each plant, an ample soil monolith was dug out and the entire rootstock was manually separated from the soil.

2.4. Sample preparation

For the ingredient analysis of the elites (Table 2) a composite sample, including rhizome, adventitious and lateral roots was investigated.

The rootstocks of the harvested clones were prepared. In the process the adventitious roots were separated from the rhizome and were allotted to a diameter category (<2 mm, 2-3 mm, 3-4 mm, >4 mm) and the rhizomes were cut into slices of 8-10 mm thickness. Each genotype was processed separately from the others. The material was carefully and accurately washed by hand and dried at 45 °C in a drying oven. The roots were dried for 21 hours, while the rhizomes were dried for 30 hours with one drying break of two hours until the residual moisture was below 12%. After drying, the roots of the different diameter categories were fractionated into adventitious roots and lateral roots. A single sample consisted of a mixture of roots of several cloned plants of the same genotype (i), a diameter category (k), such as a root type (l). Furthermore, rhizome samples of four genotypes (m) existed. These are also from several cloned plants. Thus, there are theoretically 36 samples ($n_{theoretical} = i \cdot k \cdot l + m = 4 \cdot 4 \cdot 2 + 4$). Since the root structure (thickness of adventitious roots, portions and position of lateral roots) of the genotypes distinguished, not all fractions could be generated ($n_{effectively} = 29$). Between all processing steps, the material was stored in dry and air protected boxes in a refrigeration room at 5-6°C.

2.5. Chemical analyses

The analyses to determine the ingredients valerenic acids and essential oil, as well as the determination of the water content of the drug were conducted in accordance with the guidelines of the European Pharmacopoeia (Ph.Eur.7.0, 2011). The ingredient contents were calculated based on the dry mass of

the respective fraction. The analysis of the essential oil had to be adjusted by using a smaller sample weight of 20 g and by adding a silicone anti-foaming agent to the mazerate to prevent it from boiling over. The current Pharmacopoeia do not longer include the content of extractives as a quality feature for valerian. However, for the pharmaceutical industry, the extract is still an important parameter. Hence, the content of extractives was analyzed in accordance with the monograph for valerian root of the European Pharmacopoeia of 1998 (Ph.Eur.3.0, 1998).

2.6. Statistical analysis

For the distribution of the ingredients in the different parts of the rootstock and in the different root diameter categories ananalysis of variance (ANOVA) and a comparison of mean values were performed. For the multiple comparisons, the conservative and for unequal sample sizes suitable Scheffe-test was used. In this assessment, the four clones are considered as part of a valerian population, so that the clones represent random samples from a population. The significance of the ingredient differences within the two morphological root structure groups were verified with an independent samples t-test. All statistical tests were referred to as significant at $p < 0.05$. The calculations were performed with the free statistical software R (version 3.1.0) and the package agricolae (version 1.2-0).

3. Results and Discussion

Comparison of elites and clones

The ingredient contents of the elites (2010) and the clones (2012) are quite similar in proportion. Even if the values of the elites and clones are not directly comparable - they were determined in different years – however, some aspects are still worth mentioning.

The high average contents of essential oil in the clones 2012 were remarkable (Table 2). The elites, grown and harvested in 2010, were processed practical using a drum washing machine. In contrast, the clones and the root fractions respectively, were washed gently by hand. The essential oil containing cells in the outer root layers, described in literature (Tschirch and Oesterle, 1900; Holzner-Lendbrandl, 1963; Kutschera, 1992), were probably not damaged. A gentle and rapid harvesting, drying and processing probably could have an effect on the ingredient contents, especially on the content of the essential oil.

Weight of the root fractions

The highest drug weight was determined in the adventitious roots, followed by the lateral roots and rhizomes (Fig. 3). On average, the adventitious roots capture 70 %; the lateral roots 20 % and the rhizomes 10 % of the total drug weight. Thereby, the adventitious roots with diameters 2-3 mm and 3-4 mm formed on average the bulk. Schunk (1962) concluded that a loss of fine roots during harvesting and processing should be essentially avoided for the conservation the essential oil. Taking into account the current knowledge, this is not the case (see in 3. Results and Discussion - Ingredient contents in adventitious roots, lateral roots and rhizomes). However, on average, 20 % of the drug weight composed

of lateral roots, so that the drug yields could be reduced. Each investigated clone showed an individual drug weight pattern, based on the morphological root structure.

The as thick rooted classified genotypes 710237c and 710323c, developed as only one, roots of more than 4 mm in diameter. Although, this has not been investigated systematically in the current investigation, thicker lateral roots have been observed in the clone 710323c. The high drug weight of the lateral root fraction at the clone 710323c can likely be attributed to this.

Ingredient contents in adventitious roots, lateral roots and rhizomes

In the present investigation, high contents of valerenic acids and essential oil were located in the adventitious roots, followed in descending order by the lateral roots and the rhizomes (Fig. 4 A-B). The three rootstock components differ significantly, shown by the high significant p-values for the contents of valerenic acids and the essential oil (Table 3 I.). The rhizomes, which are part of the dried root drug, have distinctly lower contents of valerenic acids and essential oil in comparison to the roots (compare with the same letter marked mean values in Fig. 4 A-B).

In contrast, the rhizomes contain on average, but with no statistically secure, the higher contents of extractives. Indeed, the extractives are currently not specified in the present European Pharmacopoeia monograph; however, they are important for the pharmaceutical industry. Due to their small share of the total root mass (see in 3. Results - Weight of the root fractions) a dilution or an accumulation effect for the ingredients should be regarded as low.

In principle, reports show a similar picture of the distribution of essential oil in the rootstock, as did the present investigation. Generally, it is not easy to understand which root type and root thickness the authors address with their descriptions. The fact that different contents of essential oil can be detected in different parts of the rootstock was also shown by Eisenhuth (1956) and Schunk (1962). Eisenhuth (1956) reported the general presence of essential oil in strong roots, fine lateral roots and rhizomes; thereby the contents decline in the order of the listed components. Schunk (1962) detected high contents of essential oil in the finest roots (under 1 mm).

Ingredient contents according to different root diameters

Fig. 5 shows the ingredient contents of four diameter categories. One category contains the values of the adventitious roots and the appertaining lateral roots. It seems as if the average contents of valerenic acids, essential oil and extractives increase slightly with the diameter categories < 2 mm, 2-3 mm and 3-4 mm. In the category > 4 mm the ingredient contents differ. The valerenic acids content increase again, the content of essential oil decrease and the extractives content remains approximately the same. However, statistically significant differences could not be observed (Table 3 II.) If the adventitious roots and the lateral roots were evaluated separately, statistically significant differences between the roots diameters could also not be detected. Therefore, a representation is omitted. The breeding goal (see in 1. Introduction - Importance of the localization of the ingredients in the root geometry) was to create new varieties of valerian with coarser rootstocks, combined with good ingredient contents. The initially

assumed negative relationship (Heuberger and Penzkofer, 2013), between the adventitious roots thickness and the ingredients contents could not be detected.

Comparison of groups with similar root structure

Studies, compared several genotypes are rarely. The most authors investigated rootstocks of one valerian population. Hörner (1989) carried out a comparative study of essential oil contents in two valerian populations. In current study, two morphological different groups (thin and thick rooted), containing two different genotypes, were investigated.

Indeed, each clone presented an individual distribution pattern of the investigated ingredients within its rootstock; however, it was possible to subdivide the clones in two groups, with a similar distribution pattern (Fig. 6 A-B). At this juncture, the first group was formed by the thin rooted clones 710177c and 710209c; the second group was formed by the thick rooted clones 710237c and 710323c (compare Table 1). The thin rooted group was characterized by high content differences in valerenic acids and essential oil between the adventitious roots and the lateral roots (Table 4 I.) The thick rooted group shows no significant differences between the root types. In both groups, the extractive contents did not or marginally differ between the two root types (Table 4 I. and Fig. 6 C) but the groups vary significant between the roots types (Table 4 II.) The contents of extractives were marginally lower in the first group than in the second group. In the rhizomes, the contents of valerenic acids and essential oil, seems to be different between the thin and thick rooted groups (Table 4 and Fig. 7); however, statistically secure only for the valerenic acids. The extractives contents do not differ.

Although, the clone 710237c and 710323c do not originate from the same genetic pool (see in 2.1. Plant material), the distribution patterns of the two genotypes in the second group look quite similar. It seems as if there might be a connection between the root morphology and the distribution pattern of the ingredients.

4. Conclusion

The study was initiated due to a 2008 started breeding work. Contrary to previous assumption, this study shows that in the investigated plant material the thicker roots contain approximately the same ingredient contents. This is a very important perception. Otherwise, the breeding goals – creation of varieties with coarser rootstocks, combined with good ingredient contents - have had to be adapted.

Furthermore, the study gives information, in which rootstock components the ingredients valerenic acids, essential oil and extractives are located. It could be shown that in the adventitious roots, the lateral roots and the rhizomes, depending on the investigated ingredient, different contents are located. In tendency, the roots contain higher ingredient contents than the rhizomes. In this study, the roots formed the bulk of the root drug, so that rhizomes may not have an influence of the root drug ingredient content.

The high proportion of lateral roots could lead the breeding focus to not reduce the proportion of lateral roots, but to increase the thickness of them; in particular, in valerian genotypes with less contents differences between the adventitious and lateral roots.

The results seem to be also advantageous for the growers. The high proportion of lateral roots causes the speculation that the loss of root mass must be prevented. However, the distribution of the ingredients exhibited that a loss of lateral roots during the harvesting and the preparation are not expected to cause a decline of the ingredient contents.

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3.2 Figures and Tables

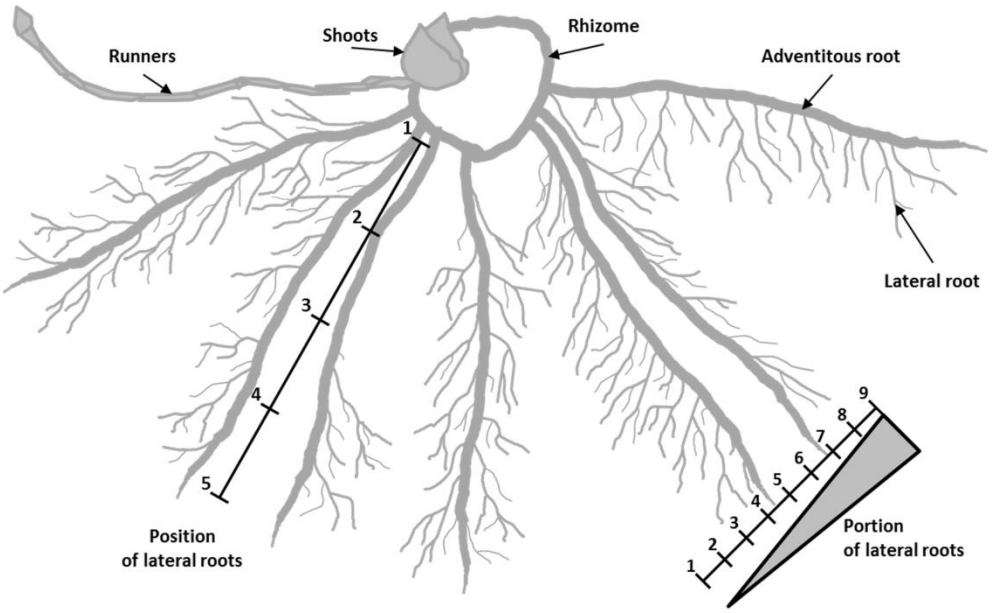

Fig. 1: Illustration and description of the investigated components of valerian rootstock. Visualization of the rating levels for the portion of lateral roots (1-9, scarce-numerous) and the position of the lateral roots (1-5, nearer-further to rhizome).

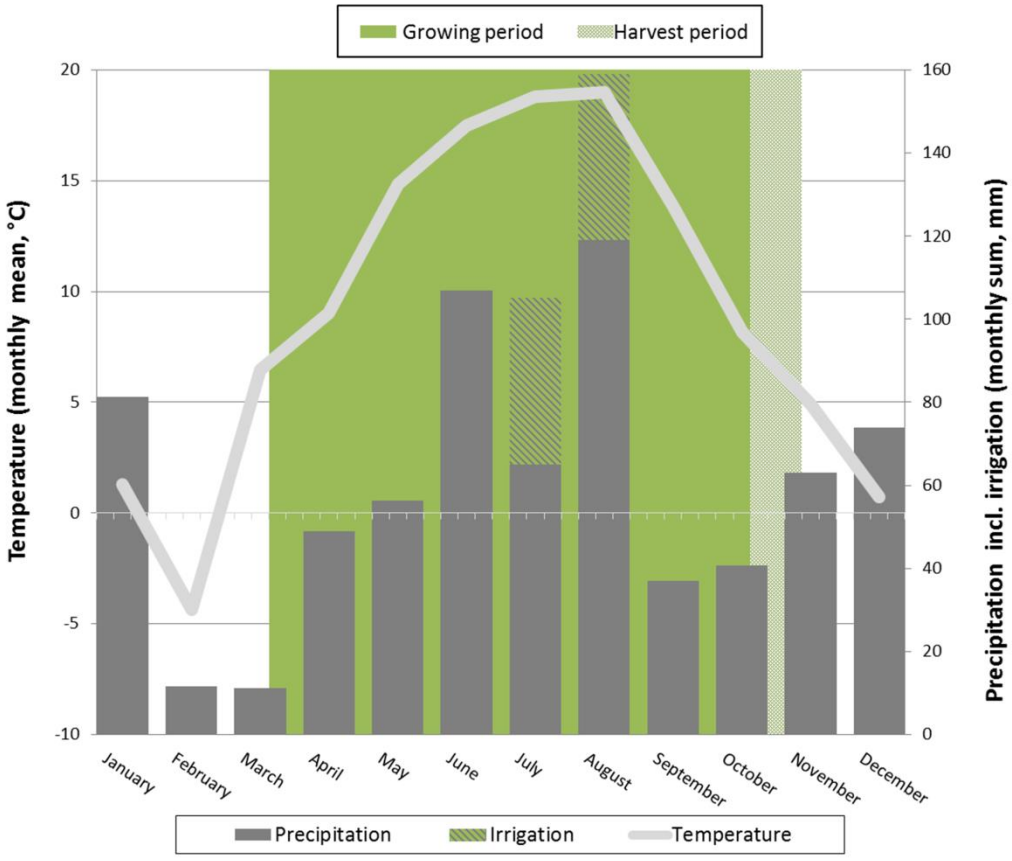

Fig. 2: The monthly mean of temperature and the monthly sum of precipitation and irrigation in 2012. Data from AgrarMeteorologie Bayern (2014).

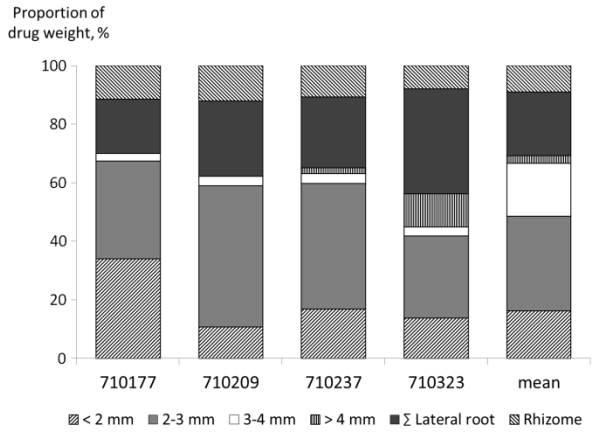

Fig. 3: Proportions of adventitious roots of varying diameters, the sum of lateral roots and the rhizomes contributing to the complete drug weight of four valerian clones.

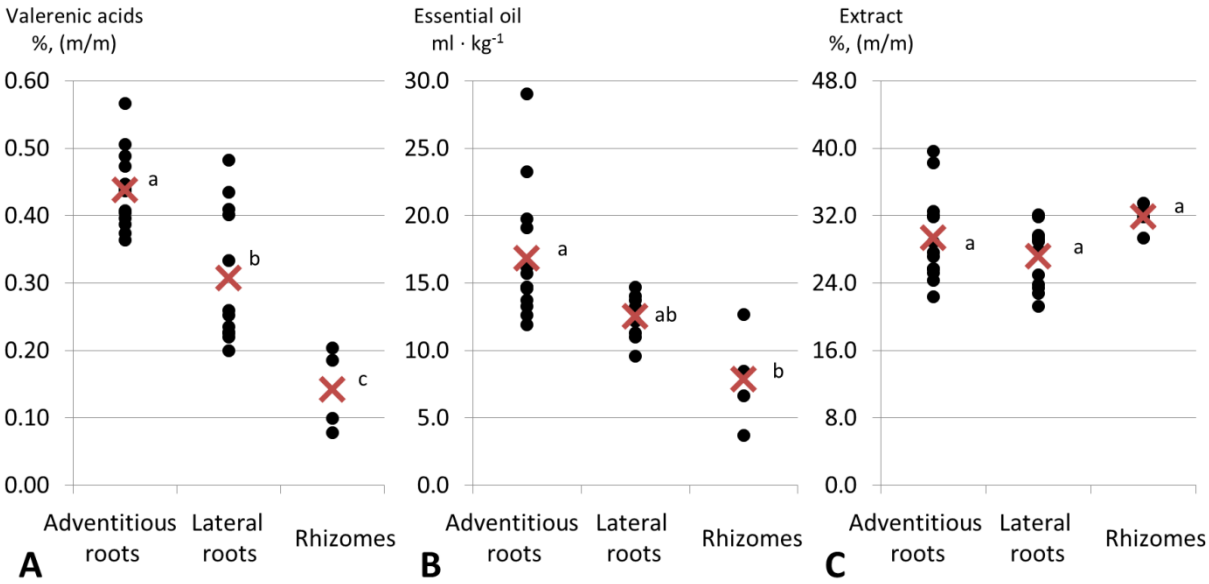

Fig. 4: Comparison of the contents of valerenic acids, essential oil and extractives in the adventitious roots, lateral roots and rhizomes of valerian clones. The x-cross gives the entire mean of adventitious roots, lateral roots and rhizomes. Mean values, which are marked with the same letter, are not significantly different ($p < 0.05$, Scheffe-test).

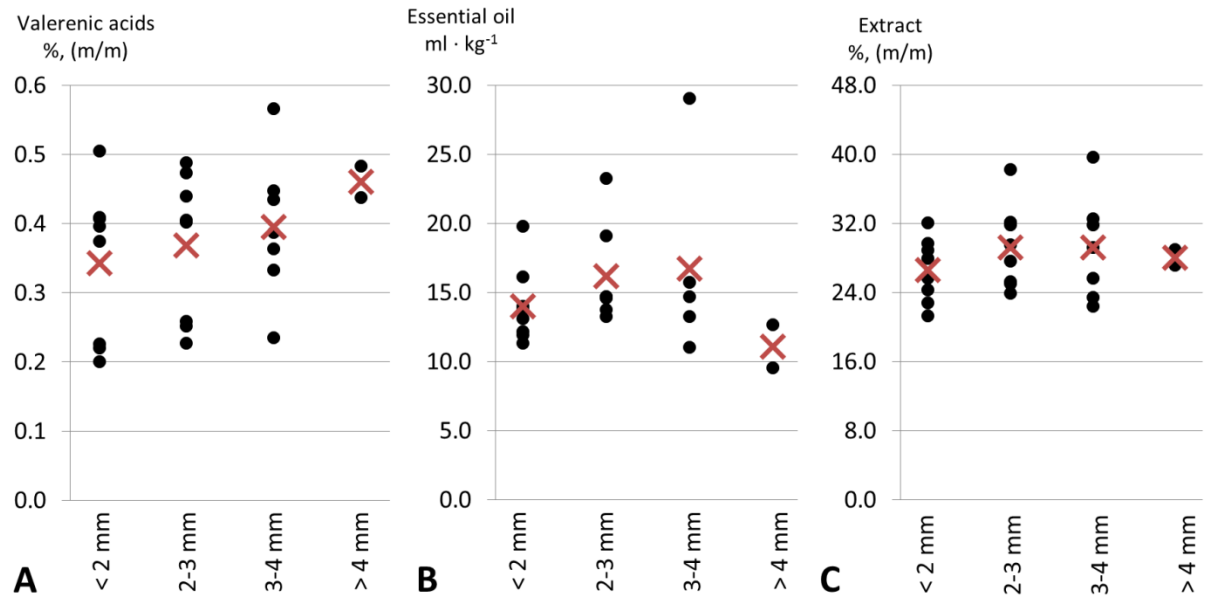

Fig. 5: Comparison of the contents of valerenic acids, essential oil and extractives in four diameter categories of valerian clones. The x-cross gives the entire mean of the root diameter < 2 mm, 2-3 mm, 3-4 mm, > 4 mm. The mean values are not significantly different ($p < 0.05$, ANOVA).

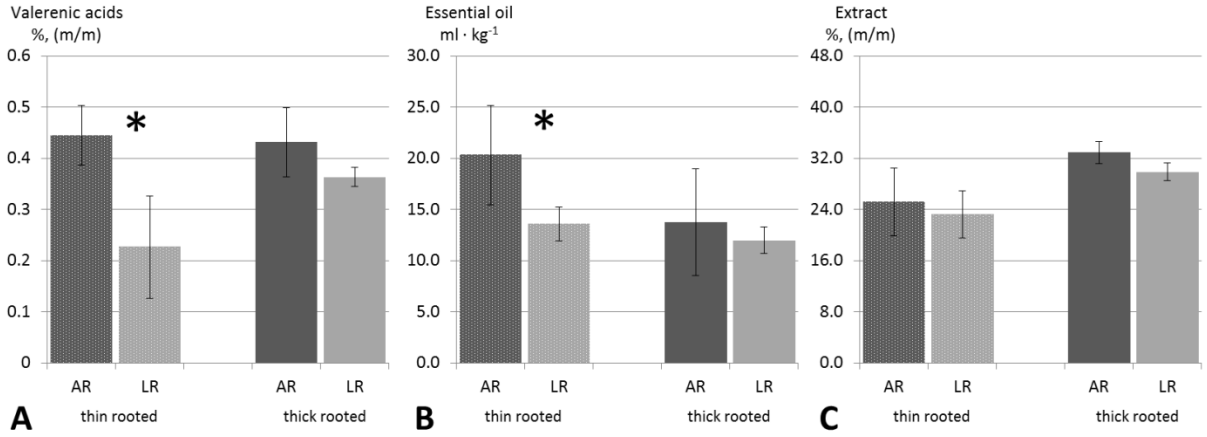

Fig. 6: Means of the contents valerenic acids, essential oil and extractives, respectively, of the adventitious (AR) and lateral (LR) roots of two groups of valerian clones with a different morphological root structure (thin rooted and thick rooted). Root types in *-marked groups are significantly different ($p < 0.05$, t-test). Vertical lines: standard deviation.

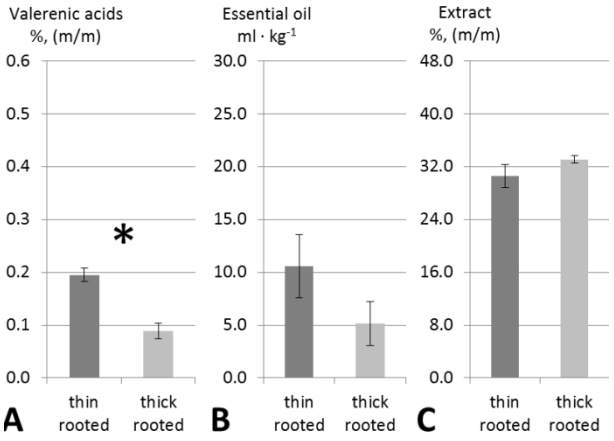

Fig. 7: Means of the contents valerenic acids, essential oil and extractives in the rhizomes of two groups of valerian clones with a different morphological root structure (thin rooted and thick rooted). The thin rooted and thick rooted groups which are significantly different ($p < 0.05$, t-test) are *-marked.

Table 1: Root morphological quality and contents of relevant ingredients in the root drug of the elites. The rating levels were awarded after harvesting on single plants.

Cultivar (Source of supply)	**Elites**	**Morphologic root structure** Classification	**Thickness of adventitious roots** Rating [A)]	**Portion of lateral roots** Rating [B)]	**Position of lateral roots** Rating [C)]
'Anton' (NLC)	710177	thin rooted	4	3	5
'Anton' (NLC)	710209	thin rooted	4	8	3
'Anton' (NLC)	710237	thick rooted	7	7	3
'Lubelski' (PHS)	710323	thick rooted	6	5	3

Seeds source of supply:

NLC: 2009, N.L. Chrestensen Erfurter Samen- und Pflanzenzucht GmbH, Witterdaer Weg 6, 99092 Erfurt, Germany

PHS: 2008, PHARMASAAT Arznei- und Gewürzpflanzensaatzucht GmbH, Str. am Westbahnhof 4, 06556 Artern, Germany

[A)] Rating levels (1-9, thin-thick); rating levels derived of a reference sample with rating level 5;
consequent 3 = 1.0-1.4 mm, 4 = 1.5-2.0 mm, 5 = 2.1-2.4 mm; 6 = 2.5-2.7 mm, 7 = 2.8-3.1 mm.

[B)] Rating levels (1-9, scarce-numerous); see Fig. 1

[C)] Rating levels (1-5, nearer-further to rhizome); see Fig. 1

Table 2: Contents of relevant ingredients in the root drug of the elites and clones. The values of the clones are mean values from all fractions from this study. The values of the elites were determined from composite samples, including roots and rhizome.

	Valerenic acids %, (m/m)	**Essential oil** ml·kg^{-1}	**Extract** %, (m/m)
Elites			
710177	0.28	8.9	19.50
710209	0.33	10.4	19.70
710237	0.18	5.0	25.30
710323	0.37	6.9	22.70
Clones			
710177c	0.31	14.1	24.95
710209c	0.33	19.9	25.53
710237c	0.29	12.9	33.87
710323c	0.41	11.3	29.86

Table 3: Comparative study (ANOVA) of valerenic acids, essential oil and extractive contents in three rootstock components (adventitious roots, lateral roots, rhizomes) and four diameter categories (< 2 mm, 2-3 mm, 3-4 mm, > 4 mm).

I. Rootstock components: Adventitious roots (AR), Lateral roots (LR), Rhizomes (RI)

	AR-Mean (SD)	**LR-Mean (SD)**	**RI-Mean (SD)**	**p-Value**
Valerenic acids %, (m/m)	0.438 (0.058)	0.307 (0.100)	0.142 (0.062)	< 0.001
Essential oil ml · kg^{-1}	16.82 (4.863)	12.54 (1.657)	7.866 (3.760)	0.001
Extract % (m/m)	29.33 (5.282)	27.14 (3.687)	31.83 (1.806)	0.165

II. Diameter category: < 2 mm, 2-3 mm, 3-4 mm, >4 mm

	< 2 mm Mean (SD)	**2-3 mm Mean (SD)**	**3-4 mm Mean (SD)**	**>4 mm Mean (SD)**	**p-Value**
Valerenic acids %, (m/m)	0.342 (0.112)	0.368 (0.106)	0.395 (0.103)	0.460 (0.032)	0.510
Essential oil ml · kg^{-1}	14.02 (2.773)	16.19 (3.663)	16.74 (7.086)	11.10 (2.182)	0.370
Extract % (m/m)	26.57 (3.690)	29.19 (4.811)	29.25 (6.040)	28.06 (1.290)	0.663

SD: Standard deviation

Table 4: Comparative study (t-test) of valerenic acids, essential oil and extractive contents in adventitious roots, lateral roots or rhizomes, respectively, in the thin and thick rooted root morphological groups.

I. Root types: Adventitious roots (AR) and lateral roots (LR) in the

	thin rooted group			**thick rooted group**		
	AR-Mean (SD)	**LR-Mean (SD)**	**p-Value**	**AR-Mean (SD)**	**LR-Mean (SD)**	**p-Value**
Valerenic acids %, (m/m)	0.445 (0.07)	0.227 (0.02)	< 0.001	0.432 (0.05)	0.364 (0.09)	0,132
Essential oil ml · kg^{-1}	20.34 (5.21)	13.62 (1.32)	0.024	13.80 (1.31)	12.00 (1.63)	0,055
Extract % (m/m)	25.15 (1.74)	23.27 (1.38)	0.076	32.91 (4.57)	29.90 (1.53)	0,140

II. Root morphological group: Thin rooted (TN) and thick (TK) rooted

	Adventitious roots			**Lateral roots**		
	TN-Mean (SD)	**TK-Mean (SD)**	**p-Value**	**TN-Mean (SD)**	**TK-Mean (SD)**	**p-Value**
Valerenic acids %, (m/m)	0.445 (0.07)	0.432 (0.05)	0.701	0.227 (0.02)	0.364 (0.09)	0.008
Essential oil ml · kg^{-1}	20.34 (5.21)	13.80 (1.38)	0.027	13.62 (1.32)	12.00 (1.63)	0.169
Extract % (m/m)	25.15 (1.74)	32.91 (4.57)	0.003	23.27 (1.38)	29.90 (1.53)	<0.001

III. Root morphological group: Thin rooted (TN) and thick (TK) rooted

	Rhizome		
	TN-Mean (SD)	**TK-Mean (SD)**	**p-Value**
Valerenic acids %, (m/m)	0.195 (0.013)	0.089 (0.015)	0.018
Essential oil ml · kg^{-1}	10.57 (2.990)	5.166 (2.074)	0.186
Extract % (m/m)	30.58 (1.771)	33.08 (0.607)	0.271

SD: Standard deviation

4. Characterization of essential oil distribution in the root cross-section of *Valeriana officinalis* L. s.l. by using histological imaging techniques

Article information:

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	Wrote the paper
Contribution of other authors:	Andrea Baron performed 100 % of experiments in her bachelor thesis
	Annette Naumann, Andrea Krähmer assisted the work and the interpretation of the Fourier-transform infrared (FTIR) imaging and co-wrote the sections, which included the FTIR.
	Hartwig Schulz and Heidi Heuberger enabled the investigation in the working groups each and contributed to writing the paper

4.1 Manuscript

Abstract

Background

The essential oil is an important compound of the root and rhizome of medicinally used valerian (*Valeriana officinalis* L. s.l.), with a stated minimum content in the European pharmacopoeia. The essential oil is located in droplets, of which the position and distribution in the total root cross-section of different valerian varieties, root thicknesses and root horizons are determined in this study using an adapted fluorescence-microscopy and automatic imaging analysis method. The study was initiated by the following facts:

- A probable negative correlation between essential oil content and root thickness in selected single plants (elites), observed during the breeding of coarsely rooted valerian with high oil content.
- Higher essential oil content after careful hand-harvest and processing of the roots.

Results

In preliminary tests, the existence of oil containing droplets in the outer and inner regions of the valerian roots was confirmed by histological techniques and light-microscopy, as well as Fourier-transform infrared (FTIR) spectroscopy. Based on this, fluorescence-microscopy followed by image analysis of entire root cross-sections, showed that a large number of oil droplets (on average 43 % of total oil droplets) are located close to the root surface. The remaining oil droplets are located in the inner regions (parenchyma) and showed varying density gradients from the inner to the outer regions depending on genotype, root thickness and harvesting depth.

Conclusions

Fluorescence-microscopy is suitable to evaluate prevalence and distribution of essential oil droplets of valerian in entire root cross-sections. The oil droplet density gradient varies among genotypes. Genotypes with a linear rather than an exponential increase of oil droplet density from the inner to the outer parenchyma can be chosen for better stability during post-harvest processing. The negative correlation of essential oil content and root thickness as observed in our breeding material can be counteracted through a selection towards generally high oil droplet density levels, and large oil droplet sizes independent of root thickness.

Keywords: Valerian, medicinal plant, root slice, thin-section, oil droplet, fluorescence-microscopy, Fourier-Transform infrared (FTIR) spectroscopy, Nile Blue A, sudan-III

1. Background

Valerian (*Valeriana officinalis* L. s.l.) is an herbaceous perennial plant with a huge variability regarding habitus, composition of ingredients, and agro-economic traits. The leaves usually are imparipinnate, and the leaflets, weakly to strongly serrated. For blooming, a vernalization is necessary and hence, the first inflorescence usually develops in the second year of cultivation. Valerian occurs on sporadically wet

habitats in the temperate zone of the northern hemisphere. This indicates that a secure water supply is necessary for cultivation. Usually, the rootstock forms a dense meshwork of thin roots (Heuberger et al., 2012b).

For medicinal purposes, the entire root system including the rhizome is used (Ph.Eur.9.1, 2017). Preparations based on valerian roots are used against restlessness and sleep disturbances (ESCOP, 2009). In Germany, the dried root of valerian is a component of about 86 phytopharmaceutical and homeopathic preparations. In North America (USA, Canada, Mexico), due to other admission procedures, more than 1,000 products with valerian root are obtainable. In Germany alone, the demand for dried roots amounts to app. 1,000 tons, equal to a market size of app. 4 Mio.€ (FNR, 2013; FNR, 2014; LNHPD, 2018; COFEPRIS, 2016; NIH, 2018).

To counteract the losses of root mass and secondary compounds during harvesting, cleaning and the further production process of dried valerian roots, breeding was started in 2008 to develop new varieties of valerian with a coarser root-system (thicker adventitious roots) and with high contents of secondary compounds. A coarser root system would probably preserve the secondary compounds, essential oil and valerenic acid (Heuberger and Penzkofer, 2017). According to the European Pharmacopoeia, the minimum content of essential oil must be 4 ml * kg-1 and of valerenic acid at 0.17 % (m/m) (Ph.Eur.9.1, 2017). The most frequent major constituents of essential oil of Valeriana officinalis L. s.l. are the monoterpenes borneol and its esters, bornyl acetate and bornyl isovalerate (Stoll and Seebeck, 1957; Stoll et al., 1957; Reichling et al., 1994; Houghton, 1997; Bos, 1997; WHO, 1999).

In contrast to the abundant analyses of pharmaceutical secondary compounds and their medicinal values, there are relatively few studies related to the physiology and localization within the root. Zacharias (1879) described essential oil to be located in "[…] the outer exodermis […]", whereas Tschirch and Oesterle (1900) found it in the "[…] single-row hypodermis […]". Both authors described one 'oil droplet' in a single exodermis cell. Localization of essential oil only in the outer cell layers of the valerian roots would support the two following observations made during the breeding of coarse valerian: (i) Considering 200 selected plants (elites), the essential oil content decreased with the increase of root thickness (Heuberger and Penzkofer, 2017). This behavior is explainable, because with increasing root diameter, the root surface area decreases in relation to the root volume (calculated as cylinder). (ii) After careful hand-harvesting and hand-processing, high essential oil contents were achieved (Penzkofer et al., 2014). Due to careful handling, the surface was not damaged and the essential oil, close to the root surface, still present. The presence of essential oil close to the root surface was confirmed by Holzner-Lendbrandl (1963) und Fridvalszky (1957), who additionally recognized small round bodies named 'oil sacs' in the parenchyma of the roots. These 'oil sacs' were found predominantly in the outer parenchyma. Violon et al. (1983) identified 'oil droplets' also in the inner parenchyma. All previous investigations remain vague about the oil droplet identification and distribution across the cross-section. In addition, they do not give information concerning oil droplets among different varieties, at different root diameters on the same plant, or at different positions along the roots.

The application of various vibrational spectroscopy methods for visualizing secondary metabolites in different plant tissue is already described for e.g. polyacetylenes and carotenoids in carrots, or essential oil components in fennel, chamomile and curcuma (Özparpucu et al., 2017; Baranska et al., 2005; Victor et al., 2017; Gonzalez-Torres et al., 2017; Baranska et al., 2004). The Fourier-transform infrared FITR imaging method allows one to study the occurrence and distribution of a wide range of molecules in cell tissues. However, it has not yet been applied for the essential oil in valerian. The fluorescence-microscopic method is suitable for the visualization and localization of secondary compounds in plant roots, and was used with sunflowers and mountain arnica (Li et al., 2012; Pljevljakušić et al., 2012; Kromer et al., 2016). Furthermore, a spectral-sensitive camera could make more oil droplet structures visible, or make chemical differentiation possible, respectively (Schultz et al., 2001; Kuska et al., 2015).

Our intention was to give a more detailed histochemical description within the valerian roots. The development of an appropriate method to visualize and clearly identify the essential oil droplets required several consecutive steps grouped into two fields: (k) Verification of oil droplets and (kk) generation of an essential oil distribution map. Verification of the oil droplets (k) was done by light-microscopic imaging and subsequent confirmation of the essential oil in the found oil droplets by Fourier-transform infrared (FTIR) imaging. Based on the results of these investigations, fluorescence-microscopic imaging for generating oil droplet maps (kk) could be applied.

Based on this, the study at hand was carried out to better understand the histochemical background of the two observations (i) and (ii). Observation (i) must be well interpreted to assess the achievability of the breeding target of a thick root-system with good essential oil content. Understanding the histological background of observation (ii) may explain why the entire essential oil is not all lost during a more robust, mechanized root harvesting and processing in large-scale valerian field production. We postulated that a great part of the essential oil droplets occur in the inner parts of the valerian root.

2. Material and Method

Plant material

In 2010, three elite plants were selected from the variety 'Anton' (seed source: N.L. Chrestensen Erfurter Samen- und Pflanzenzucht GmbH, Erfurt, Germany, 2008) and one elite plant from the variety 'Lubelski' (seed source: PHARMASAAT Arznei- und Gewürzpflanzensaatzucht GmbH, Artern, Germany, 2009) based on their differing root morphology and essential oil contents (Table 1). Two elites were characterized as thin-rooted, meaning that they predominantly formed a highly branched and felted rootstock with thin adventitious roots. The other two elites predominantly formed a chunkier and less felted rootstock with thicker adventitious roots; these were called thick-rooted.

In contrast to common cultivation, in the current study, clones were used in order to have plants with known analytical and identical genetic backgrounds. Plants of seed propagated valerian populations would probably vary too much in the contents of secondary compounds (Eisenhuth, 1956). Therefore,

the four elite plants were cloned by sterile micropropagation using inflorescences at an early bud stage as starting tissue and side shoots as propagation parts. Rooted plantlets were cultivated for two years in the field of the experimental station Baumannshof of the Bavarian State Research Center for Agriculture (48° 42' N / 11° 32' E, 360 m MSL). A detailed description of the plant material, the cloning by *in-vitro* propagation and the cultivation conditions are described in Penzkofer et al. (2014), where the same plant material was used.

Due to the age of the plants, flowering was induced and shoots started to develop in spring of the harvest year. The inflorescence development influences the essential oil content and causes a decrease of the essential oil content from summer to autumn (Strazewicz, 1971). Therefore, the inflorescences were cut off in an early stage of development to counteract the decline (Eisenhuth, 1955). Our plant material was harvested in autumn (2014), the usual harvest time for valerian cultivation. At least one cloned plant of each elite was dug out carefully and the adventitious roots were separated into four diameter fractions (< 2 mm, < 3 mm, < 4 mm, > 4 mm; Table 1). The fresh roots were stored in air-tight containers at 5-6 °C to prevent dehydration of the roots and a loss of essential oil. Prior to preparation for microscopy, the roots were washed carefully with water.

Imaging methods

Verification of oil droplets (k)

Classic light-microscopic imaging

To confirm literature observations we used the classic method for microscopic imaging by fixing the cell components with a formaldehyde-propionic acid-ethanol-solution (5%-5%-90% FPA) and embedding the fixed roots in historesin (2-hydroxyethyl methacrylate). The starch was colored with a Lugol's solution (potassium tri-iodide) and washed out with potassium hydroxide (KOH).

Thin slices of embedded roots, as well as thin longitudinal-section slices of root parenchyma and exodermis from fresh valerian roots, were colored with sudan-III-solution to make the lipids visible (Johansen, 1940). The sections were evaluated with a light microscope (ZEISS Axiostar plus, Carl Zeiss AG, Oberkochen, Germany) at magnification of 200.

Fourier-transform infrared (FTIR) imaging

Thin-section slices of 0.01 mm thickness were made by a freezing microtome (Leica CM 1100, Leica Biosystems Nussloch GmbH, Nussloch, Germany). Each slice was screened for colorless round bodies by light microscopy that was integrated in the FTIR spectrometer. Sections with round bodies were subsequently analyzed applying FTIR imaging. FTIR transmission spectra of the thin-section slices were recorded with the FTIR spectrometer Varian 4100 FTIR (Agilent, Waldbronn, Germany) combined with the IR-microscope Varian UMA 600 (Agilent, Waldbronn, Germany). The thin-section slices were placed on a ZnSe window and FTIR images were produced with a 32 x 32 focal plane array detector (FPA). The spectra were recorded over the wavelength range of 4,000 to 850 cm^{-1} and 128 scans per spectrum were accumulated.

The absorption band at 998 cm^{-1} represents mainly cellulose, whereas the signal at 1737 cm^{-1} was assigned to C=O vibration of bornyl acetate. Both signals were used to display the distribution in pseudo-color images.

Fluorescence-microscopic imaging and mapping of differing root material (kk)

Sample preparation and producing of thin root-slices

From each cloned elite (CE) and root fraction (RF), two well-developed fresh roots with the typical root appearance were chosen and classified into three approximately 60 mm long parts, called horizons (HZ1 to HZ3, Figure 1). HZ1 represents the rhizome-near part, which would certainly be harvested after cultivation, and HZ3 represents the rhizome-far part, which probably remains in the ground. Along the whole length of the horizons, several thin-sections were taken and prepared.

The cutting of thin root-slices was done by hand with a height adjustable cylinder-microtome. A segment of the respective root diameter fraction and horizon was clamped between a buffer-material, cut out from a carrot root parenchyma. This material has a comparable structure and consistency to the examined valerian roots. Thereby, the fresh valerian roots were well enclosed. With a moderate pressure, a constant velocity and without displacement, a straight metallic blade was moved through the valerian root tissue. The cut was performed in a constant angle of 20 degrees. Through the moveable and height adjustable hanger, thin root-slices of a uniform thickness of 0.2 mm were able to be produced. These root-slices were then placed on top of a drop of water on a microscope slide. From each horizon, 15 to 20 thin-sections were cut, but just a low number of slices (seven on average) were suitable for the following image processing, analysis and evaluation.

Staining and fluorescence-microscopy

After the root-slices were cut, the water was removed and, based on the experiences of Fridvalszky [21], one to three drops of 1 % aqueous solution of Nile Blue A (Carl Roth GmbH und Co. KG, Karlsruhe, Germany) were applied. After incubating the root-slices for one minute at room temperature, the solution was carefully removed. A drop of water was applied and the root-slices were covered with a cover glass. A further incubation period of at least 30 minutes followed.

The fluorescence-microscopy was done with a magnification of 100 (ocular 10x, objective 10x, ZEISS Axiostar plus, Carl Zeiss AG, Oberkochen, Germany). The initial light generated by a HBO50 high pressure mercury arc lamp was filtered for excitation at 430-510 nm and for emission at 475-575 nm. The images were taken with the connected digital camera ZEISS AxioCam ERc 5s (a hyperspectral camera was not available) and instantly transferred to the connected computer. Due to the limited lens coverage, the final picture of a complete root-slice had to be composed of several partial images.

The green fluorescence oil droplets were imaged with a high-contrast against the black background. Despite all precautions taken, the root-slices did not always have exactly the same thickness and the cytoplasm of the cut cells was more or less leaked, so that the light transmittance of the cell

layers varied among and within the root-slices. In order to make the oil droplets clearly visible in all areas of the root slice, brightness and contrast were adjusted through the camera software (ZEISS AxioVison, Carl Zeiss AG, Oberkochen, Germany) by one to two units, upwards or downwards, for each image. The depth of focus on the microscope was not changed so that the same cell layer was shown on each image.

Image processing

All partial images of one root slice were converted from the camera software's own file format to the compressed free TIFF file format and then manually merged to one complete root-slice image with the image editing program Adobe Photoshop CS6 (Adobe Systems Software, Dublin, Republic of Ireland).

The composed root-slice images were analyzed with the image analysis software ImageJ (Schneider et al., 2012). Software macros were developed to generate black-white-masks of the oil droplets, the root-slice center and the root-slice edge from each composed root-slice image (Figure 2-A, Figure 2-B, Figure 2-C). More information on the functionality is given in the additional file 1.

The principle steps were to convert the composed image of the root slice into 8 bits grayscale image, to reduce the background noise using ImageJ image filtering operators (median, dilate), and segment the oil droplet by using the Huang Threshold method implemented in ImageJ.

Due to the variable position and the inconsistently round shape of the central cylinder, as well as the edge of the root-slice, the center of the root-slice was manually marked in the original composed root-slice image. After treating images that way, they were converted into a binary image (black and white) and x-y-coordinates of the now black particles were determined by using filters for particle size and particle circularity. The determination of the x-y-coordinates of the centers of the root slices and each point of the edges were done in a similar manner. Each image was treated with the same adjustments.

Data evaluation and statistical analysis

For data evaluation, the determined x-y-coordinates were adapted and related to each other. The coordinates of the centers were used as new coordinate origins (formula Ia and Ib) and the distances of the oil droplets and the edges related to the center were calculated with formula II. This was only achievable when the center, the oil droplet and the corresponding point of the root edge all lay on an imaginary line. An example is shown in Figure 3-A. The corresponding point of the root edge was calculated with help of the polar angle, which must be the same for the distance between the center and the oil droplet (CD in Figure 3-A) and for the distance between the center and the corresponding point of the edge (CE in Figure 3-A). The polar angle of both distances was calculated with Formula III. At last, the relative distances of the oil droplets and the root edges to the center were determined (Formula IV). This data was used for the evaluation.

The relative distance data was assigned to one of nine classes with a class width of 11.11 %; this led to the interval limits for class 1 = [0 % - 11.10 %); class 2 = [11.11 % - 22.21 %); class 3 = [22,22 %

- 33,32 %); class 4 = [33,33 % - 44,43 %); class 5 = [44,44 % - 55,54 %); class 6 = [55,55 % - 66,65 %); class 7 = [66,66 % - 77,76 %); class 8 = [77,77 % - 88,87 %); class 9 = [88,88 % – 100 %]. Figure 3-B shows a generalized illustration of the nine classes. Class 1 was always within the central cylinder; class 2 delineated mostly the border of the central cylinder. The area of both classes together was addressed as central cylinder. The classes 3 to 8 comprised the parenchyma, class 9 the outer cell layers. The area of the classes increased from the center to the edge. Therefore, both the number of oil droplets in each class and the oil droplet density were determined to compare the classes. The density was calculated as the quotient of number of oil droplets over the class area.

For statistical analysis, in each class, the mean number of oil droplets of the root-slices was determined. The distribution of these oil droplets as affected by the horizons, the root diameter fractions, the root classification and the genotypes, were compared with the Friedman-Test and Wilcox-Test. To compare the different factor levels, such as different genotypes (CE), root diameter fractions (RF) or horizons (HZ), the oil droplet density for each root-slice was calculated. An analysis of variance (ANOVA) and t-Test was performed. For all statistical analyses, significance was given at $p < 0.05$.

Unless otherwise described, the following data represent the mean values over the other factors and their factor levels.

Formula:

Ia: $x_{mod} = x_i - x_C$

Ib: $y_{mod} = y_i - y_C$

II: $r = \sqrt{x_{mod}^2 + y_{mod}^2}$

III: $\phi = \arccos\left(\frac{x_{mod}}{r}\right)$

IV: $pp = \frac{(r_{CD} * 100)}{r_{CE}}$

x_{mod}, y_{mod} = according to the new origin coordinate modified x- and y-coordinate

x_i, y_i = x- and y-coordinates of oil droplets and root edges, respectively

x_C, y_C = x- and y-coordinates of centers

r = distance (radius) in pixel points

ϕ = polar angle

pp = relative distance

r_{CD} = distance (radius) from center to oil droplet

r_{CE} = distance (radius) from center to root edge

3. Results

Classic light-microscopic imaging

Figure 4-A shows the colored cross-sections of the root parenchyma of a randomly chosen valerian root. Between the colored grains of starch, colorless round bodies are visible. It is assumed that these colorless round bodies contained essential oil (Figure 4-A).

In thin cross-sections and longitudinal-section slices of fresh roots, only few colorless round bodies were stained red by the application of the sudan-III-solution (Figure 4-B and 4-C).

Fourier-transform infrared (FTIR) imaging

Figure 5 presents the light-microscopic picture of a thin valerian root slice and the corresponding FTIR images obtained by integration of the absorbance at 1737 cm^{-1} and 998 cm^{-1} (top, from left to right). Whereas the signal at 1737 cm^{-1} can be tentatively assigned to bornyl acetate, the absorption at 998 cm^{-1} mainly represents cellulose matrix. The spectrum presented in the bottom of Figure 5 was taken at the crossed lines shown in the integration map for 998 cm^{-1} (Figure 5, top left). As indicated by arrows in the light microscopic picture, two intact oil bodies might be identified due to the intensive absorption at 1737 cm^{-1}. The red colored part in the right upper corner of that image might be the result of destroyed oil bodies and smeared essential oil due to microtome preparation of the root slide. Generally, in the sections used for FTIR imaging, it was very rare to find intact colorless bodies for which high absorbance around 1737 cm^{-1} was observed. Unfortunately, the presence of oil bodies could not be confirmed by adjacent staining experiments.

Fluorescence-microscopic imaging and mapping of different root material

A total of 678 root-slices of valerian were evaluated. The number of root-slices was distributed quite evenly among the different levels of the factors: cloned elites (CE), root diameter and classification, root horizon (HZ). However, fewer root-slices were analyzed in regard to the root diameter fraction RF4, because the thin-rooted cloned elites did not form roots with a diameter greater than 4 mm (Table 1, Table 2).

The distribution of the mean number of oil droplets in each factor level was approximately constant (Figure 6). Significant differences between the distributions of the factor levels of root classification (Wilcox: $p = 0.012$), root diameter fraction and horizon (Friedman: $p < 0.001$ and $p = 0.005$, resp.) were especially visible in class nine (Figure 6-B to C). In general, the mean number of oil droplets increased from the center to the outer cell layers. The central cylinder was almost free of oil droplets, whereas, the parenchyma included 57 % (42-64 %) of the mean number of oil droplets on average. In the outer cell layer, completely covered by class nine, 43 % (36-56 %) of the mean number of oil droplets were present, on average.

To consider the varying areas of the classes, the oil droplet density was a more suitable parameter to compare the classes than the number of oil droplets. A constant density was not found. Similar to the mean numbers, the density of oil droplets also rose up from class one to class nine (Figure 7). Each factor showed a significant effect on the density of oil droplets (Friedman: CE $p = 0.014$; RF $p < 0.001$; HZ $p = 0.016$; Wilcox: root classification $p = 0.012$).

The root diameter of each factor level affected the number of oil droplets. Thicker root-slices contained more oil droplets, as presented in Table 2. This meant, ultimately, that the number of oil droplets increased with root thickness, root diameter and in the upper located root horizons as compared to the lower horizon. As shown for the class comparison, the density of oil droplets allowed for a better comparison between factor levels. Among the cloned elites, CE2 showed the highest oil droplet density (ANOVA $p < 0.001$; Figure 8-C). RF1 was the root diameter fraction with the highest oil droplet density

(ANOVA $p < 0.001$; Figure 8-C), whereas each horizon showed a significantly different oil droplet density (ANOVA $p < 0.001$; Figure 8-D), with an increasing oil droplet density from HZ1 to HZ3.

Figure 8-A shows fluorescent oil droplets in a representative root cross-section. Based on the image area of the oil droplets, the size of oil droplets allocated to classes 3 to 7 was approximately 72 % larger than the size of the oil droplets allocated to classes 1 and 2. This was more or less recognizable for all root-slices, but was not examined in detail. In the outer cell layer of young lateral roots we could already find oil droplets (Figure 8-B).

4. Discussion

Histochemical structures and methods (k, kk)

Comparable histological structures were found as reported for valerian in literature

We compared our breeding plant material with the existing literature information by using classic light-microscopic imaging techniques. Many grains of starch are densely arranged in the cells and distributed over the entire parenchyma (Holzner-Lendbrandl, 1963; Fridvalszky, 1957; Kutschera, 1992). In longitudinal-section slices from fresh roots, oil droplets were visible, who are located near the cell-wall (Holzner-Lendbrandl, 1963; Szentpetery et al., 1966). Large-lumen cells of the exodermis were observed, however, the filling of the cell with one oil droplet (Holzner-Lendbrandl, 1963; Fridvalszky, 1957; Kutschera, 1992) could not be detected.

The staining of the round bodies with sudan-III-solution worked well in the fresh roots, but not in the embedded roots. Probably, the essential oil volatilized due to either the use of ethanol during the embedding process or due to the ethanol-containing sudan-III-solution itself. Similar effects were observed by Fridvalszky (1957) and Violon et al. (1983), who concluded hereupon that the lipid bodies contained 'volatile oil'.

The colorless round bodies are filled with essential oil

Generally, FTIR imaging can provide information about the distribution of different plant constituents without destructing the plant constituent containing cell structure. These constituents include lipids, carbohydrates, and lignin, as well as secondary metabolites, e.g. terpenoids (Schulz et al., 2014).

Nevertheless, the local accumulation of the strong absorbance at 1737 cm^{-1} tentatively assignable to bornyl acetate indicates an essential oil distribution in distinct cellular structures. As bornyl acetate is a principal component of the essential oil of valerian, the results from FTIR imaging confirms the theory that essential oil is located within the colorless bodies. However, only very few intact oil droplets were found in the thin cryo-sections. The preparation of thin root slides of a thickness below 0.01 mm usually resulted in disruption of the oil bodies and smearing of the essential oil. Therefore, the results of FTIR imaging have to be seen as basic studies combining visual images of the sample with chemical information. The authors' own previous studies on valerian root sections performed with FT-Raman spectroscopy did not deliver additional information about essential oil distribution. Only the

differentiation of various root tissue mainly based on carbohydrate profile was obtained and with the instrument used, the local resolution was limited to around 150 µm (data not shown).

Modern Raman microscopes achieve a local resolution below 1 µm depending on the laser and aperture used. To gather suitable signal intensity, the Raman laser needs to be exactly focused, which demands for the extremely thin root slices. With application of the necessary laser power, the brownish root material often started burning and the spectra showed high fluorescence appearance. A reduction in laser power resulted in a lack of signals. Therefore, if the analytes don't contain Raman active bonds (e.g. C-C double bonds in carotenoids) and the sample is colored (as the majority of plant derived material is), Raman spectroscopy investigations are technically challenging.

In recent years, another vibrational spectroscopy method gained attention in plant analysis. Hyperspectral (near infrared) spectroscopy imaging can be used for various analytical purposes as species identification, disease detection or nutrient quantification, but is focused on macroscopic samples due to the relatively coarse spatial resolution of several 100 µm (Manley et al., 2009; Türker-Kaya and Huck, 2017; Liu et al., 2015).

Taking into account the above described characteristics of each method, FTIR imaging in combination with subsequent staining to affirm the preliminary results, seems to be the most favorable analytical strategy. The resulting FTIR distribution maps showing the strong C=O vibration at 1737 cm^{-1} confirm the results of staining experiments and fluorescence-microscopy.

Due to the failed repeatability of the FTIR imaging of oil droplets, the more robust method of fluorescence-microscopy was chosen to visualize the oil droplets.

Relative distances and class width are suitable to compare the root diameter fractions and root horizons

The oil droplet position was calculated as relative distance from the root center to the root edge. The relative distances were allocated to nine width classes, which allowed a comparison between the root diameter fractions and root depth (horizons), regardless of the real root diameter of the considered root slice. The root slices seldom showed a circular shape. Often, the root slices were oval or had coves. An absolute and constant class width would not have been appropriate, because the border of class nine would not necessarily be congruent with the edge of the root slice. Further information concerning the oil droplet distribution would be achieved if densities of the classes are calculated based on absolute diameters and cross-section areas. For our purposes, the absolute oil droplet densities of the total cross-section areas were sufficient.

Meaning of oil droplet distribution and density for valerian breeding and production (i, ii)

With the current investigations, we were able to show that oil droplets can be found in the whole parenchyma. These results are in good agreement with previous studies of Violon et al. (1983) and Szentpetery et al. (1966). Besides the identification of the oil droplets, the number and density of oil

droplets are also now available for different varieties, different root diameters on the same plant or for different positions along the roots.

Conclusions about the relationship of oil droplet occurrence and essential oil content of genotypes are limited

Chemical-analytical data does not exist for the currently studied plant tissue, but genetically identical plant material has been investigated analytically by Penzkofer et al. (2014). We tried to compare and re-estimate the results of both studies.

The comparison of the four clones gives only limited information for the relationship between oil droplet occurrence and oil content, due to the low number of clones, the limited genetic variability among clones and the environmental and year's effect, which all potentially influence the essential oil content (Heeger, 1956; Bernath, 1997). Even Penzkofer et al. (2014) could only detect trends in this regard, but identified clone CE2 as the one with the highest essential oil content. This did not coincide with our essential oil mapping data. Concerning the number of oil droplets per root cross-section, clone CE3 shows the highest values, followed by clone CE4 and CE2 (Table 2). Thus, the number of oil droplets was not a suitable identifier for the essential oil content. This may be due to the varying oil droplet sizes in the inner parenchyma.

In contrast, the essential oil droplet density based on total root cross-section area is more informative. Clone CE2 had the highest oil droplet number/mm² (Figure 7) and it showed the highest oil content in the study by Penzkofer et al. (2014). However, to evaluate the relationship between essential oil droplet density and essential oil content, more data is needed.

The number and density of oil droplets are not negatively related to the root thickness

The mapping results showed an expected increase of the number of oil droplets per cross-section from thinner to thicker roots (Table 2). For the fractions with low to high root diameters (RF1-RF3), the number of oil droplets was in accordance with the essential oil content results of Penzkofer et al. (2014). The fraction with the largest root diameter (RF4) showed the highest number of oil droplets in our study, but the lowest essential oil content in their study. It has to be noted that the essential oil content was determined by a very small number of data points and comprised only the clone CE4, because the other clones did not develop roots of this diameter. Considering CE4 only, the oil droplet number was the same for thick and very thick roots (RF3 and RF4, respectively).

The oil droplet density of total root cross-sections was highest in the thin roots (RF1) and lower in medium to thick roots (RF2-4). Thus, no linear relationship between oil drop density and root thickness can be derived. The high oil droplet density in thin roots may partially be an effect of root age, assuming that a higher portion of young roots occurred in this root fraction, and that oil droplets are formed at a very early stage of development, a phenomenon we were able to see with our image of a young lateral root. However, root age may not entirely explain the difference in oil droplet density between RF1 and RF2 as there was no difference between RF2 and RF3.

Concerning the breeding target of thicker roots, we found a positive relationship between root thickness and number of oil droplets per root cross-section. We did not find a negative relationship between root thickness and oil droplet density, especially when medium to very thick roots were considered. Consequently, there is a potential for selection of thick roots that have high oil droplet densities and, therefore, also likely high essential oil content. This is supported by the experience that many inbred lines with coarse roots and high essential oil content could be derived from clone CE4.

Oil droplet distribution should be considered in cultivation, harvesting and breeding

Considering that 43 % of the oil droplets were localized in the outer section of the roots (class nine), it is evident that a careful root harvest and processing, without damaging the root surface, is important. This also means that a high portion of oil droplets are located in the inner parenchyma, information which is important for valerian cultivation, as well as for breeding selection.

Heindl and Hoppe (2010) reported that intense and long washing of valerian roots leads to a loss of secondary compounds. Therefore, the more essential oil is stored in the inner parenchyma, the lower should be the losses during the processing, and the higher is the processing stability of the roots.

One of the main breeding targets in the past was increasing the essential oil content (Bernath, 1997). With this target in mind, the losses of oil during processing and storage are minor. We now show that in different single plants, represented by the clones of elites, different essential oil distributions exist. Choosing plant types with steadily increasing oil droplet density curves instead of plants with exponentially increasing oil droplet densities towards the root edge implies that a higher portion of the oil will be located in the inner parenchyma. Moreover, processing stability and possibly, also, storage stability of valerian roots can be increased. Our imaging and mapping method, in combination with the calculated density curves, can serve as a selection tool for identifying suitable plants.

The comparison of horizons provides further information about the reliability of the production process. The horizons represent harvest depths: The greater the depth, the thinner the formed roots, and the greater the likelihood that they will be more easily lost during the harvesting process.

These horizons contain a lower number of oil droplets. Thus, the harvest of the root parts in deeper soil layers does not have to be exhaustive in order to obtain high essential oil contents in the root drug, especially when 96 % of the root mass is located in the topmost 10 cm of the ground (Neumaier, 2017).

5. Conclusion

For the first time, the essential oil distribution in entire valerian root cross-sections of different varieties, root thicknesses and root horizons were visualized, applying an imaging and mapping fluorescence-microscopy technique. The applied methods of FTIR spectroscopy and fluorescence-microscopy allowed for the determination of oil droplets as clearly differentiated structures.

Our results give insight into the cross-sectional essential oil distribution at valerian harvest time. Although the existing natural variability and the limited plant material investigated in this study only

allow for a brief overview, some aspects derived from the results could be used for further investigations and for future breeding work:

- The number, density and distribution of essential oil droplets of genetically different plant material vary and allow for the selection of suitable plant material for breeding purposes, independent of root thickness.
- The breeding plant material should generally exhibit a high oil droplet density level as this is one of the factors for the essential oil content.
- A high and homogeneous oil droplet density should be aspired in the inner parenchyma in order to avoid essential oil losses during harvesting and post-harvest processes.

It still remains unclear which oil droplet characteristic is preferred as a selection trait. In consequence, a compromise between the absolute oil droplet density and the oil droplet density curve must be found, while at the same time, considering root thickness and individual genetic background.

Finally, a careful, but not necessarily exhaustive harvest, as well as careful post-harvest procedures, confirmed as best methods for conserving the essential oil and obtaining a high quality of the valerian root drug.

Authors' contributions

MP performed the preliminary investigations, adapted the fluorescence-microscopy technique, designed the experiment and analyzed the data. AB performed the fluorescence-microscopic works, including sample preparation, staining and image processing, and was a major contributor in writing the manuscript. AN and AK assisted the work and the interpretation of the Fourier-transform infrared (FTIR) imaging and co-wrote the sections, which included the FTIR. HS and HH enabled the investigation in their working groups. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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4.2 Figures and Tables

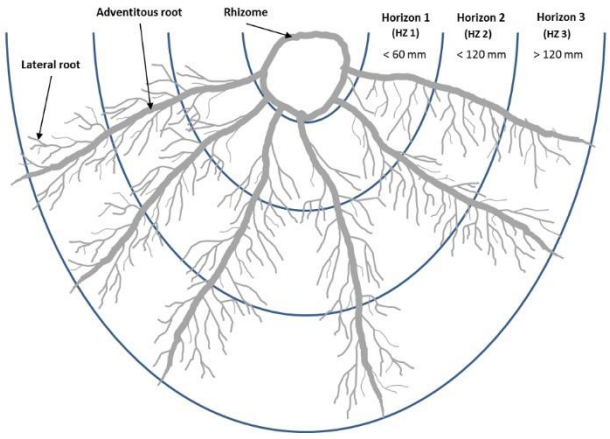


Fig. 1: The valerian root system components and visualization of the investigated horizons.

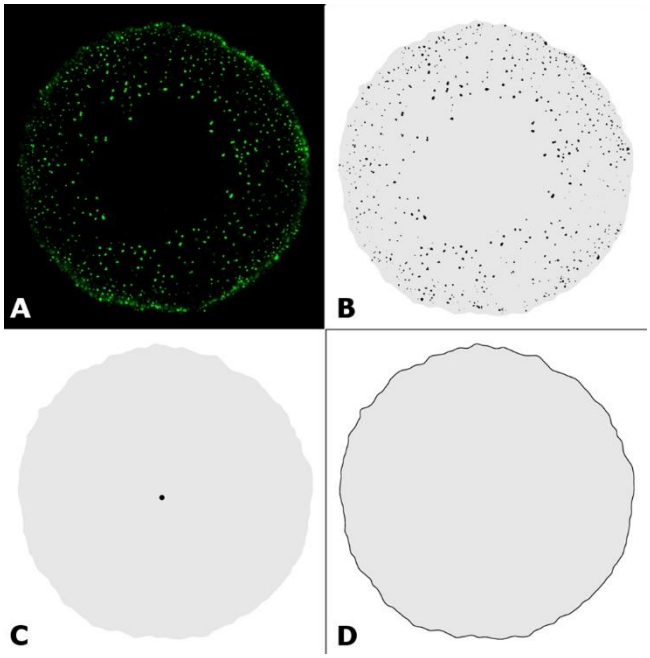


Fig. 2: Different black-white-masks derived from the original composed root-slice image. A: Root-slice image with green shining oil droplets. B-D: Black-white masks of B: the oil droplets, C: the center of the root slice, D: The edge of the root-slice. The x-y-coordinates of the oil droplets and the center (black particles in B and C) and each point of the edge in D, and the size of the grey area (seen in B, C, D) were determined automatically by the image analysis software.

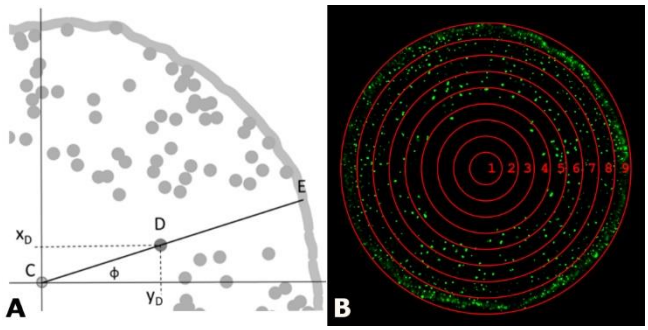


Fig. 3: A: Schematic illustration of a root-slice segment with the identified elements center (C), oil droplets (D, labeled is one oil droplet) and the root edge (E). ϕ indicates the polar angle with C as pole and the x-axis (horizontal line) as polar axis. B: Generalized illustration of the nine classes (1 to 9), to which the oil droplets (pp, Formula IV) were assigned based on their relative distance to the center.

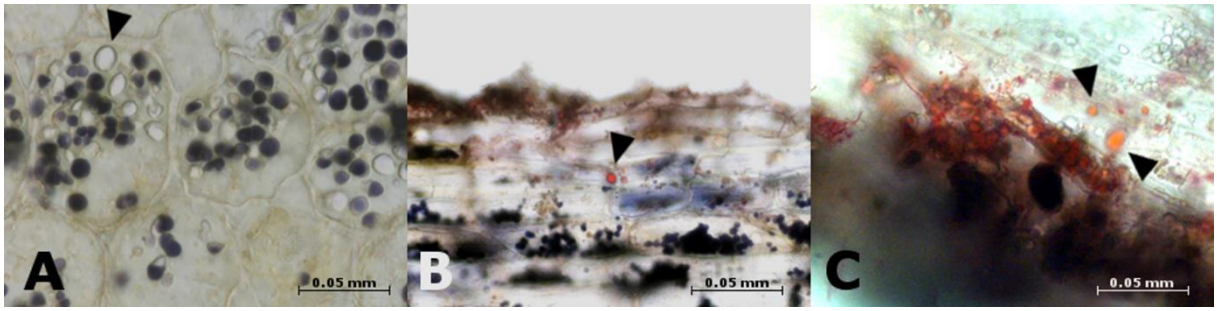


Fig. 4: Microscopic images of thin-section slices from fixed and embedded (A), and fresh (B + C) valerian roots. A: Cross-section through the root parenchyma with stained starch (Lugol's solution) and intermediary colorless round bodies (►). B + C: Longitudinal-section of the root parenchyma and exodermis. In addition to the colored starch, the red-colored bodies, which were stained by use of a sudan-III-solution, are visible (►).

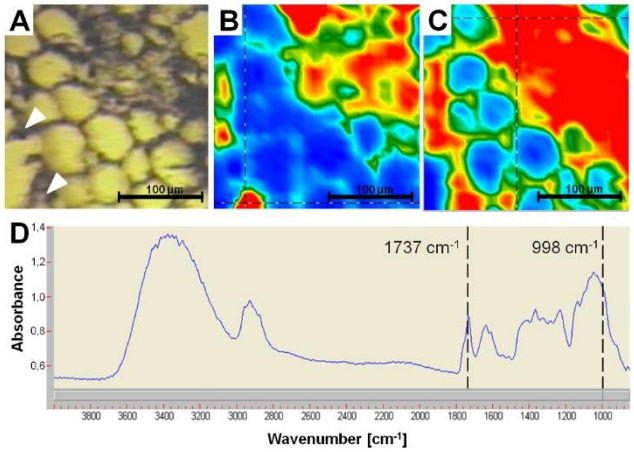


Fig. 5: Light-microscopic (A) and FTIR images, of valerian root cross section showing high absorbance for mainly bornyl acetate at 1737 cm^{-1} (B) and cellulose at 998 cm^{-1} (C) The spectrum was taken from the cross mark in the appropriate FTIR images for cellulose (D). Colors blue, green, yellow, and red represent increasing content – the warmer the color, the higher the spectral intensity.

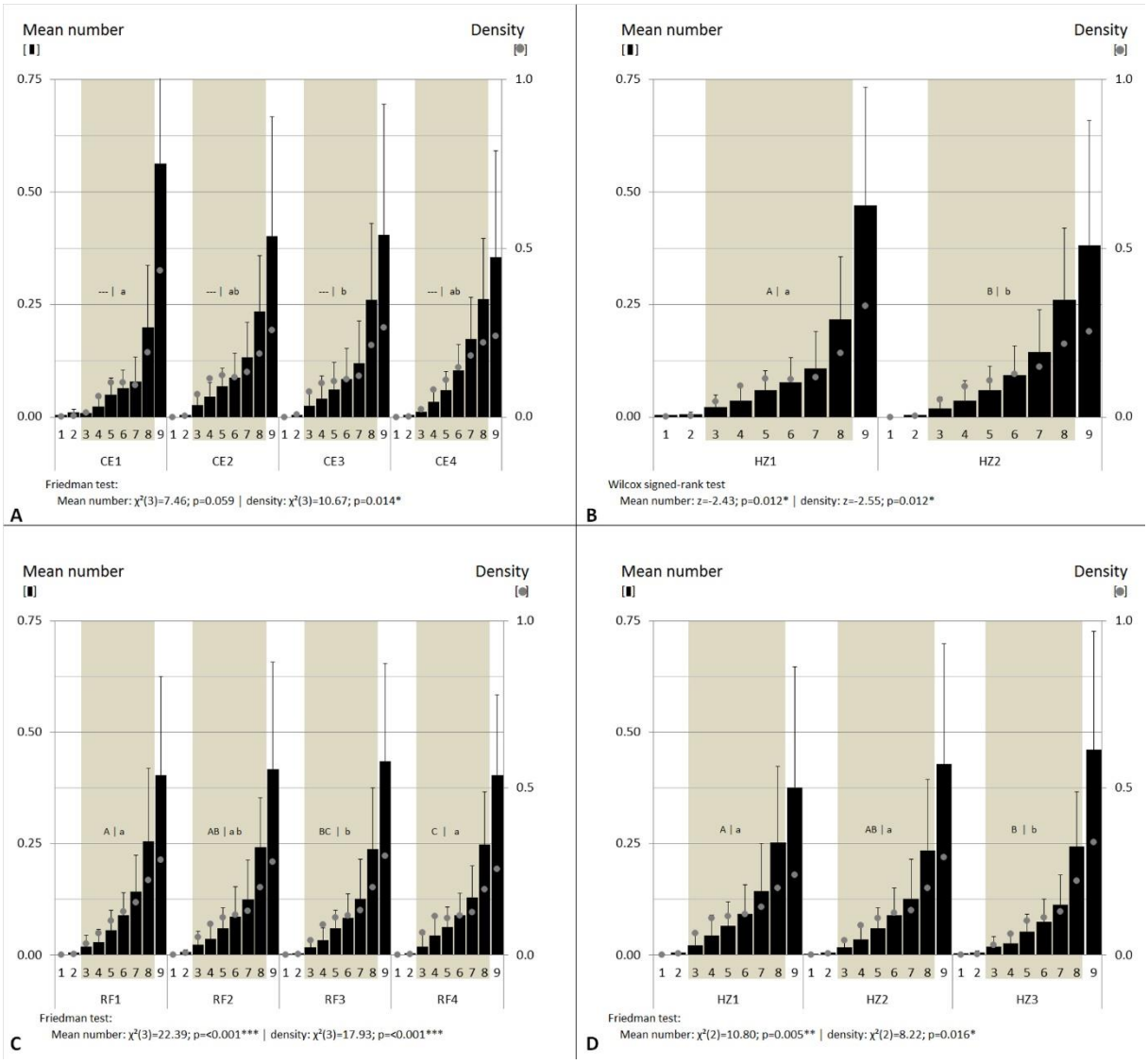


Fig. 6: Mean number (columns) and density (points; mean number/class area) of oil droplets, determined from root thin section slices of valerian and allocated in nine classes (1 to 9). Shown are four cloned elites (CE1, CE2, CE3, CE4) (A), two root classifications (thin- and thick-rooted) (B), four root diameter fractions (RF1, RF2, RF3, RF4) (C) and three root horizons (HZ1, HZ2, HZ3) (D). Vertical lines: standard deviation. The same capital and small form letters mark that the oil droplet mean numbers and the densities are equal ($p < 0.05$, SNK), respectively. The colored background marks the classes of the parenchyma. Further details of the cloned elites, the root classification, the root diameter fractions, the horizons and the parenchyma are shown in the text.

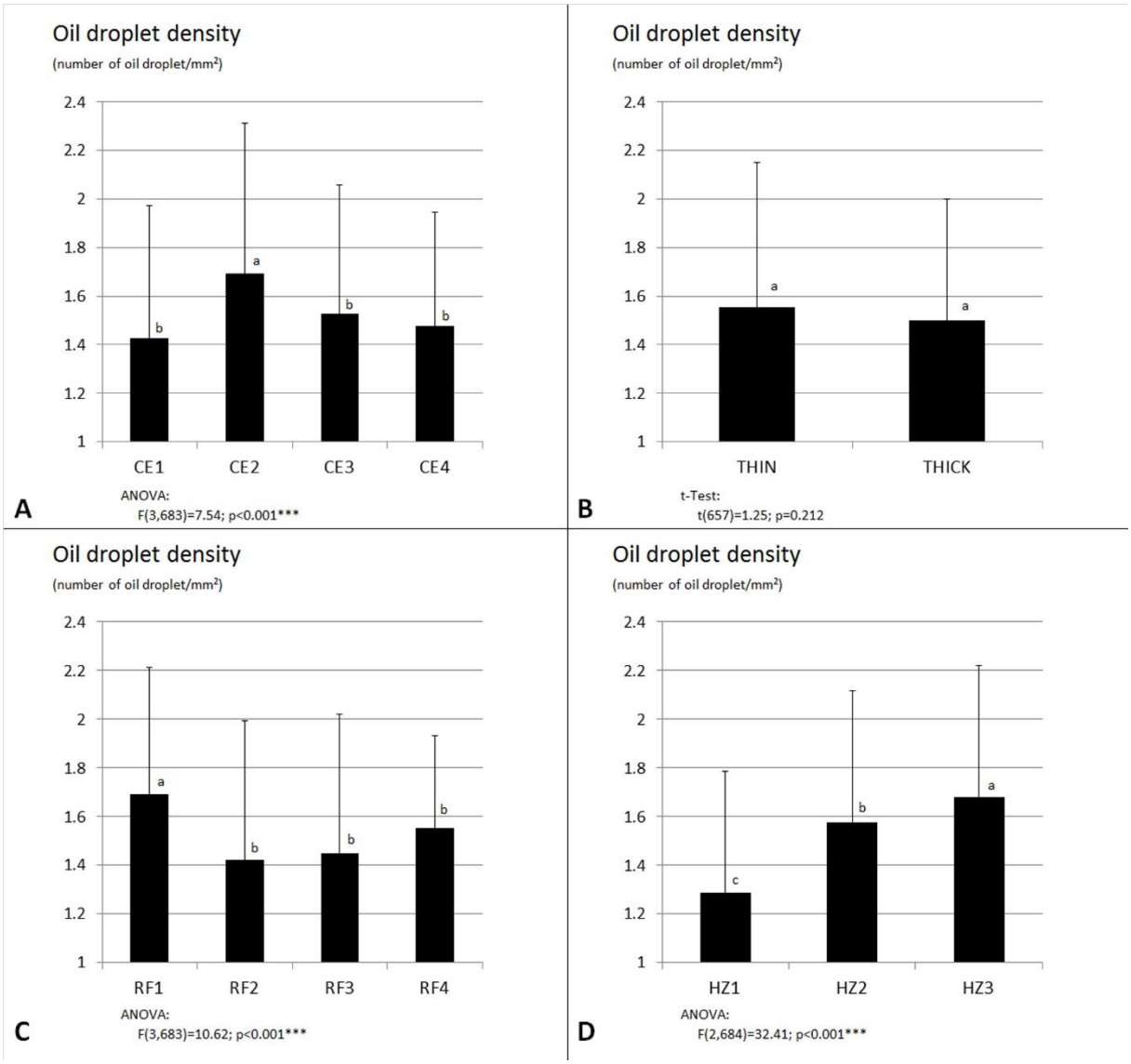


Fig. 7: Mean oil droplet density (number of oil droplets/mm²), determined from root thin section slices of valerian. Shown are four cloned elites (CE1, CE2, CE3, CE4) (A), two root classifications (thin- and thick-rooted) (B), four root diameter fractions (RF1, RF2, RF3, RF4) (C) and three horizons (HZ1, HZ2, HZ3) (D). Further details of the cloned elites, the root classification, the root diameter fractions and horizons are shown in the text. Vertical lines: standard deviation. Different letters mark significant differences between the oil droplet densities ($p < 0.05$, SNK). Number of root slices given in Table 2.

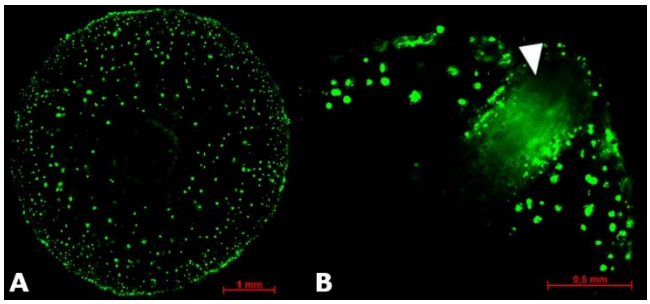


Fig. 8: Fluorescent oil droplets in cross-sections of valerian roots: Adventitious root (A), growing lateral root breaking through the exodermis (marked with ▹)

Table 1: Root morphology types and content of essential oil in the root drug of the cloned elite plants (CE). Prevalence of root thickness fractions (RF) in the clone plants.

Cloned elites	Varieties	Morphological root structure	Essential oil [A)]	Root fraction (diameter)			
		Classification	ml kg^{-1}	RF1 (< 2 mm) [B)]	RF2 (< 3 mm) [B)]	RF3 (< 4 mm) [B)]	RF4 (> 4 mm) [B)]
CE1	'Anton'	thin-rooted	8.9 (14.1)	●	●	●	—
CE2	'Anton'	thin-rooted	10.4 (19.9)	●	●	●	—
CE3	'Anton'	thick-rooted	5.0 (12.9)	●	●	●	●
CE4	'Lubelski'	thick-rooted	6.9 (11.3)	●	●	●	●

[A)] Essential oil content of the mother plant (elites) and in brackets the cloned elites, determined in 2010 and 2012, respectively.

[B)] Diameter at the base (Horizon 1, see Figure 1) is decisive for the classification to a fraction. The ● indicates the formed and the — indicates the not formed fractions.

Table 2: Number of slices, sum of oil droplets and mean number of oil droplets in the root-slices of four cloned elites (CE1, CE2, CE3, CE4), of two root classifications (thin- and thick-rooted), of four root diameter fractions (RF1, RF2, RF3, RF4) and of three horizons (HZ1,HZ2, HZ3)

	Number of root-slices	Sum of oil droplets	Mean number ± standard deviation of oil droplets per root-slice
Cloned elites			
CE1	176	34665	197 ± 84
CE2	162	43466	268 ± 149
CE3	171	61419	359 ± 255
CE4	178	53666	301 ± 168
Root classification			
Thin	338	78131	231 ± 125
Thick	349	115085	330 ± 217
Root diameter fractions			
RF1	211	37422	177 ± 87
RF2	195	42647	219 ± 98
RF3	210	77602	370 ± 192
RF4	71[A)]	35545	501 ± 239
Horizons			
HZ1	206	68904	334 ± 225
HZ2	246	74994	305 ± 184
HZ3	235	49318	210 ± 107

[A)] The root diameter fraction was only formed by CE3 and CE4 (Tab. 1)

4.3 Supplemented Data (*'Additional file 1'*)

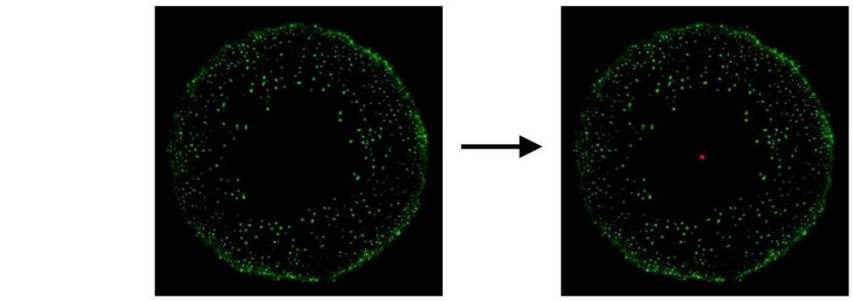
Description of analyzation steps and software macros for localization of the oil droplets, root-slice area and edges, as well as the root-slice center.

Available also online at https://static-content.springer.com/esm/art%3A10.1186%2Fs13007-018-0309-4/MediaObjects/13007_2018_309_MOESM1_ESM.pdf.

Characterization of essential oil distribution in the root cross-section of *Valeriana officinalis* L. s.l. by using histological imaging technique

Penzkofer M, Baron A, Naumann A, Krähmer A, Schulz H, Heuberger H

Analyzation steps and software macros for localization of the
① oil droplets, ② root-slice area and edges, as well as ③the root-slice center.



①

```
//*** Macro Oil Droplets ***
run("8-bit");
setThreshold(80, 255);
setOption("BlackBackground",
  false);
run("Make Binary", "thresholded remaining
black");
run("Dilate");
run("Median...", "radius=4");
run("Analyze Particles...",
  "size=350-7000 circularity=0.50-
  1.00 show=Masks display clear");
```

②

```
//***  Macro Area and Edge ***
run("8-bit");
setAutoThreshold("Huang");
setThreshold(0, 0);
run("Make Binary");
run("Invert");
run("Gaussian Blur...", "sigma=20");
setAutoThreshold("Huang");
setThreshold(1, 255);
run("Make Binary");
run("Fill Holes");
run("Create Selection");
run("Enlarge...", "enlarge=-100");
run("Enlarge...", "enlarge=100");
setBackgroundColor(255, 255, 255);
run("Clear Outside");
run("Select None");
run("Minimum...", "radius=60");
run("Create Selection");
run("Set Measurements...", "area
  centroid center perimeter feret's display
redirect=None decimal=3");
run("Analyze Particles...",
  "size=100000-Infinity
  show=Outlines display" );
```

③

```
//*** Macro Center ***
run("Split Channels");
selectWindow(img_rgb_title + "
  (red)");
setThreshold(20, 255);
run("Make Binary", "thresholded remaining
black");
run("Analyze Particles...",
  "size=500-Infinity circularity=0.80
  -1.00 show=Masks display clear");
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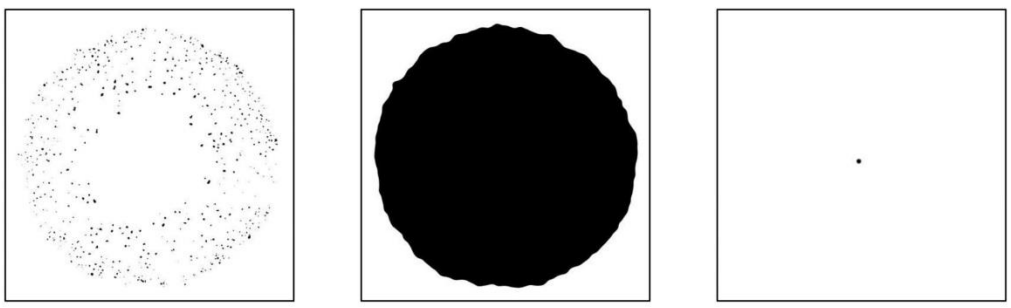

Software: ImageJ, Language: IJ1Macro

5. Estimation of outcrossing rates using genomic marker and determination of seed quality parameters in *Valeriana officinalis* L. s.l. under field conditions

Article information:

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	Wrote the paper
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	Stefan Seefelder contributed to the AFLP analysis and to writing the paper

5.1 Manuscript

Abstract

Knowledge of the biological properties of pollination and fertilization are essential for breeding and the development of breeding concepts. In this investigation, panmictically cross-pollinated seeds of two selected combining partners of valerian (*Valeriana officinalis L.*) were produced and in the progeny, the outcrossing rates (OCR) were determined by using amplified fragment length polymorphism analysis (AFLP). The previous assumption that *Valeriana officinalis* L. shows a predominantly high outcrossing rate (OCR) was confirmed. The OCR ranged from 76.5 % to 97.7 %. Several mother plants showed an OCR of 100 % in their progeny. Partially involved heterozygous marker-bands could have led to undetected outcrossings and to a lower OCR. The preferred outcrossing direction and the individual seed amount of the mother plants may influence the performance of a seed mixture, generated by both partners as mother plants.

Keywords: Panmixia, AFLP, tetraploid, seed yield, thousand-seed weight, germination capacity

1. Introduction

1.1 Plant description

Valerian (*Valeriana officinalis* L. s.l.) is an important medicinal plant. Preparations with valerian are used against restlessness and sleep disturbances (ESCOP, 2009). For medicinal purposes, the entire root system, including rhizomes, is used (Ph.Eur.9.1, 2017).

The pharmaceutically used valerian is summarized into the collective species *Valeriana officinalis* L. sensu latu [s.l] (Heuberger et al., 2012b). Predominantly, valerian forms naturally di-, tetra- and oktoploid cytotypes. The cultivated varieties and accessions are often tetraploid. Results of genotyping and ITS-region analysis can be understood as an indication for a possible allopolyploid origin, at least for the step from the diploid to the tetraploid level (Heuberger et al., 2012a).

Valerian is an herbaceous perennial plant, occurring on fresh habitats in the temperate zone of the northern hemisphere. The appearance is very variable. The leaves usually are odd pinnate with more or less pairs of weakly to strongly serrated leaflets. The inflorescences form a cyme with multitude small flowers and develop in the second year after vernalization (Heuberger et al., 2012b). Over couple of weeks, continuously, new androgynous flowers appear. Within the inflorescences all development stages of buds, flowers and seeds can be located at the same time.

1.2 Background and aim of this work

Knowledge of the outcrossing rate (OCR) is important, when choosing a breeding procedure for crop improvement and for commercial seed production.

A currently running valerian breeding project try to develop a new variety, which based on crossing of two selected parents (combining partners) (Heuberger and Penzkofer, 2017). A classical hybrid breeding procedure cannot really implemented, because of the missing male sterility in the

breeding material. A pollination control by emasculation by hand is not economical practicable due to the multitude small flowers. Other pollination control mechanisms, like the application of chemicals or the application of hot water show not a sufficient emasculation, are not permissible or are not desirable (Kempf, 1986).

Therefore, the consideration was, if the often described allogamy in valerian could use for generating hybrid seeds (seeds originated by cross-pollination) (Konovalova et al., 1978; Heuberger et al., 2012b). In the described case, the OCR would specify the extent and the tendency to cross-pollination of one combining partner (of one parent). The higher the OCR value, the higher would be the tendency of this parent to cross-pollination. This means that the progeny of this parent originated by pollination with foreign pollen. In the reverse conclusion, a low OCR value ore an OCR value of zero indicates a high percentage or a full self-pollination.

Investigations showed that mother plants will produce seeds, even when cross- or self-pollination is coerced (Konon and Korneva, 1980; Konon and Novikova, 1981). Probably, distinctive self-incompatibilities and genetically crossing-barriers does not exist in valerian. In contrast, it can be also assumed that a progeny, originated by open pollination and random mating (panmixia), is a mixture of cross- and self-pollinated descendants. Differentiated information about the outcrossing rate or the natural pollination behavior is not available. Therefore, the main aim and focus of the current investigation was to determine the ratio of outcrossing in the progeny of panmictic and open pollinated breeding plant material and to estimate the natural pollination behavior (**Q1**).

In literature, outcrossing rates are often determined for dominant/recessive morphological markers, for example the flower color in *Hibiscus sabdariffa* L. (Vaidya, 2000) and *Glycine max* L. (Ray et al., 2003), the seed coat color in poppy (Dobos, 1996), the covering with trichomes in *Lycopersicon pimpinellifolium* L. (Rick et al., 1978) or the variable sensitivity to powdery mildew in *Epilobium parviflorum* Schreb. (Simonnet and Delabays, 1993). These characteristics are simple to detect, but not useful for valerian. In valerian, the just described characteristics occur non-homologous and just very variable. The situation is similar for the contents of secondary compounds. Nabloussi et al. (2013) used the percentage of oleic acid as biochemical DNA-fragment for estimating the cross pollination in safflower. Valerian cultivars with low and high contents of secondary compounds (e.g. valerenic acid and essential oil) exist (Bomme et al., 1999), but the secondary compounds also vary from year to year. Even if cross pollinated descendants would have an intermediate content of secondary compound, it would hardly be possible to detect them. A further method, traditionally used for estimating OCR in forest-trees, is the isozyme-system (Adams, 1983). Investigations using isozyme polymorphisms in valerian were not successful (Penzkofer et al., 2014). Genome analysis methods are today often the method of choice, but there are no reports on heritability of defined DNA-fragments at valerian. The amplified fragment-length polymorphism-analysis (AFLP) is applicable without prior knowledge about the genome, and has been successfully applied to detect and estimate OCR in different species (e.g. *Mentha L.* spec. (Shasany et al., 2005), *Moringa oleifera* Lam. (Muluvi et al., 2004)). The AFLP analysis

was also successful used in valerian to identify the relationship of diverse valerian accessions and varieties (Heuberger et al., 2012a). An established procedure for estimate the OCR in valerian was not available. In the current investigation, such procedure should be developed by using the AFLP analysis for estimate the OCR (**Q2**).

During the seed production, the generated seeds can be harvested separate for each parent, or together as seedmix of both parents. In case of using the seedmix methode, beside to possible differences in the parents OCRs, also differences in several seed quality parameters, can influence the progeny seed composition. The proportion of seeds originated by cross- and self-pollination can also influence the performance of the progeny. Therefore, the seed yield, as well as the thousand-seed weight and the germination capacity of the seed of each parent was be determined (**Q3**).

2. Materials and methods

2.1 Plant material and seed production

In 2012, 10 inbreeded descendants (I_1) of 16 selected tetraploid single plants (elites) were examined for homozygously present and non-present DNA-fragments by an amplified fragment-length polymorphism-analysis (AFLP). The method is described in section 2.4 Amplified fragment-length polymorphism-analysis (AFLP). Five elites were found that differed in at least one DNA-fragment, and three pair-combinations (PC) were composed (Table 1). The donor combining partner contributed one DNA-fragment at pair-combination PC-B and two DNA-fragments at pair-combination PC-C. The combining partners in pair-combination PC-A can be acceptor as well as donor of one DNA-fragment each (in the following named as PC-A and PC-A_{rzp}).

The five chosen elites were propagated (cloned) by *in-vitro* micropropagation techniques. For the sterile micropropagation, inflorescences at an early bud stage were used. In the beginning, young and small inflorescences (0.5–1.5 cm) were removed, sterilized in 3% sodium hypochlorite for 20 min and dissected. The applied culture media were all based on the compilation of (Murashige and Skoog, 1962). The establishing media received an addition of 0.5 mg/l 6-benzylaminopurin (BAP) and the growing and rooting media received 5 mg/l BAP. The climate conditions in the growing room were regulated at a temperature of 22–24◦C and at a lighting time of 12 h per day. After acclimatization and cultivation in the greenhouse, the cloned plants were vernalized for 6-weeks at 4 °C.

After the vernalization, the plants were cultivated in a greenhouse and suitable plants were compiled according to their level of development. To exclude any somatic mutations, the chosen individual plants were analyzed for their DNA band patterns.

The chosen plants were planted at the end of April 2013 at two locations. The first location (L-1) was the experimental station Puch of the Bavarian State Research Center of Agriculture (48° 11' N / 11° 13' E, 550 m MSL), the second location (L-2) were two fields of ESKUSA GmbH in Steinach (48° 57' N / 12° 36' E, 348 m MSL). At each location, two pair combinations with an open-pollinated polycross-system (four-rows-four-columns-planting/crossing-scheme for pair-combination PC-C at location L-2 and five-rows-five-columns-planting/crossing-scheme for all other pair-

combinations at both locations) were established (Figure 1). The reduced scheme of PC-C became necessary, because the DNA band pattern of one verified combining partner shows unexpected and inexplicably fragments. The pair-combination PC-A was planted in both locations; PC-B and PC-C were planted in one location each. In this way, more pairs with a limited number of plants could be tested.

The plants of the two combining partners were planted alternately (40 cm x 40 cm), so that each plant has the combining partner plants as direct neighbors and consequently a high and evenly distributed pollination possibility with external pollen. The pair-combinations were placed with a distance of at least 200 m and not behind each other in the main wind direction. Table 1 gives an overview of the pair-combinations at the respective location.

All pair combinations flowered and developed seeds in 2014 and 2015. The seed harvesting and seed purification was carried out separately for each plant in the center ('core plot'). Usually, these are nine, at the reduced pair-combination four plants. The seeds were harvested when half of the potentially developing seeds were matured. Thereby, a loss of the first, usually better developed seeds should be prevented. The seed processing steps were: cutting of the complete inflorescence, subsequent drying at room conditions without forced ventilation, threshing, separating the vital seeds by sieving and vertical air sifting and finally storing the seeds at 5-6 °C under dark and dry conditions. The seed yield of each mother plant, the weight of 1000 pure seeds (the thousand-seed weight) and the germination capacity according the regulations of the International Seed Testing Association (ISTA, 2009) were determined.

2.2 Seedling cultivation of descendants and sample preparation

The sowing was carried out by hand for each acceptor-mother plant separately, placing one seed per pot in multipot-trays. The growing conditions were according to Heuberger et al. (2012b). From each mother plant, 32 young plants were grown. After germination, the plants were cultivated until the second to third true leaf has been developed. For analysis, 100 mg leaf material of 11 randomly selected F_1 plants from each mother plant were sampled, freeze-dried (Labonco Freeze Dry System, Kansas City) at -50 ° C for at least 48 hours and then pulverized in a ball mill (Retsch MM2000, Haan) for 2 min. The samples were stored (dry and dark) until the AFLP analysis was conducted.

2.3 DNA extraction

The DNA extraction was performed using the Invisorb® Spin Plant Mini kit (Co. STRATEC Molecular GmbH, Berlin) and included the following steps: lysis of the sample material, binding the DNA to the membrane, removal the ethanol and washing out the DNA (STRATEC, 2017). The prepared DNA was stored at -20 ° C until further analysis.

2.4 Amplified fragment-length polymorphism-analysis (AFLP)

The AFLP analysis for valerian was carried out according to Vos et al. (1995) with minor modifications, established for hop (Hartl and Seefelder, 1998; Seefelder et al., 2000) and already used for valerian (Heuberger et al., 2012a). For the second, the selective amplification step, *Eco*RI and *Mse*I primer pairs

were used carrying three selective nucleotides. The primers and targeted base sequences are listed in Table 2. The amplified products were separated on 5% polyacrylamide gels.

2.5 Data evaluation and statistical analysis

In the acceptor mother plant progeny, the number of plants with a present donor DNA-fragment was determined. The outcrossing rate (OCR) was defined as the percentage of hybrids (DNA-fragment is present) in the examined plants of the acceptor progeny. The individual OCR is the OCR of one mother plant, while the mean OCR was calculated based on individual OCRs of all mother plants in the pair-combinations, locations and years.

The significance of differences between locations, trial years, DNA-fragments and combining partners were verified with an independent samples t-test. Differences among the pair-combinations were analyzed by a Kruskal-Wallis test, because of the missing homogeneity of variance. The Scheffe-test was used for the multiple comparisons. All statistical tests were referred as significant at $p < 0.05$. The calculations were carried out using the "agricolae" package in R software (R Core Team, 2016).
The valerian plant material used in this investigation is tetraploid (Heuberger et al., 2012a). In tetraploids, each DNA-fragment inducing allele (A), can be present in four different combinations, from 1 (simplex) to 4 (quadruplex) (Schmalz, 1989; Stoskopf et al., 1993). All four allele-combinations (Aaaa, AAaa, AAAa, AAAA) are phenotypically visible (+) and show a DNA-fragment on the electrophoresis-gel image. The nulliplex allele-combination (aaaa) is not phenotypicaly visible (-) and shows no DNA-fragment. In crossed and inbred descendants, different allele-combinations/phenotypes can arise, depending on the parent allele-combination (Table 3).

3. Results

3.1 OCR

The OCRs were calculated as the ratio of found donor DNA-fragment in the progeny of the acceptor mother plant. Figure 2 shows an example DNA-fragment pattern of the reciprocal usable pair-combinations PC-A and PC-A_{rzp}.

In total, 641 plants from 60 acceptor mother plants were examined for occurring DNA-fragments. An average outcrossing rate (OCR) of 84 % was determined (Table 4). Overall, 16 of 60 mother plants showed an OCR of 100 % in their descendants (shown in supplement table). The maximum mean OCR (97.7 %) was determined for pair-combination PC-A, location L-2, trial year 2014. The minimum mean OCR (76.5 %) was determined for pair-combination PC-B, trial year 2014. The pair combination PC-B generally showed the lowest mean OCR (78.9 %), followed by PC-A_{rzp} (82.0 %), PC-C (90.7 %) and PC-A (91.6 %). Differences of the OCR between the pair-combinations existed (Figure 3; $H(3) = 12.48$, $p = 0.005$).

In pair-combination PC-A and PC-A_{rzp}, respectively, no statistical differences of the OCR between the locations and the trial year were observed. Depending on which combining partner acts as donor or acceptor, the direction of pollen and DNA-fragment transfer influence the OCR (Table 5;

t(34) = 3.10, p = 0.004). As already shown for pair-combinations PC-A and PC-A_{rzp}, also differences of the trial year were not determined in PC-B. The pollen and DNA-fragment donor plant of pair combination PC-B transfers two DNA-fragments. The determined OCR of both DNA-fragments did not differ.

3.2 Seed quality parameter

The mean value of several seed quality parameters (seed yield, thousand-seed weight, germination capacity) were calculated based on data from individual mother plants and the combining partners, locations and trial years were compared (Table 6). The total average seed yield for one mother plant was 1.90 g, the total average thousand-seed weight 0.78 g and the total average germination capacity was 86.9 %.

The maximum average seed yield (3.69 g) was determined for pair-combination PC-B, location L-1, combining partner 710217 (acceptor) in trial year 2015. The minimum average seed yield (0.77 g) was determined for pair-combination PC-A, combining partner 710323, location L-1 in trial year 2015 (shown in supplement table online). Statistical differences in the seed yield were determined between the combining partners of pair-combinations PC-A and PC-B, wherein the acceptor mother plant showed a higher seed yield each.

The germination capacity is an important seed quality parameter for valerian producers. The maximum average germination capacity (93.8 %) was determined for pair-combination PC-A, combining partner 710313, location L-2 in trial year 2014. This lead also to differences of the combining partners in just this pair-combination. A correlation between germination capacity and OCR was observed (Figure 4).

The thousand-seed weight ranged between 0.62 g and 0.99 g (pair-combination PC-B, combining partner 710125, location L-1 in trial year 2014 and pair-combination PC-A, combining partner 710323, location L-2 in trial year 2015 (shown in supplement table online). In contrast to the seed yield and germination capacity, statistical differences between combining partners were not, but differences between the trial years and the location were observed for seed germination capacity.

In comparison of the three pair-combinations, differences for thousand-seed weight (H(2) = 24.29, p < 0.001) and germination capacity (H(2) = 13.33, p = 0.001) exist (Figure 5).

4. Discussion and conclusion

In this investigation, panmictic cross-pollinations of tetraploid valerian, each with two selected combining partners, were examined for their outcrossing rate (OCR), determined by amplified fragment-length polymorphism-analysis (AFLP). Furthermore, several seed quality parameters (seed yield, thousand-seed weight, germination capacity) were determined, due to the possible influence of them to the composition of harvested seeds. Three research aims (**Q1**, **Q2**, **Q3**) were in focus. The glance was always related to an implementation on the practicable breeding work and variety seed production.

(Q1) The main aim and focus of the current investigation was to estimate the pollination behavior **(i)** and to determine the ratio of outcrossing in the progeny of breeding plant material **(ii)**.

(i) The current investigation confirmed the previous assumption that *Valeriana officinalis* L. prefers allogamy (cross-pollination) and shows this in predominantly high outcrossing rates (OCR). The observed average of the OCR lay at 84%.

The pollination with foreign pollen is supported by the time-delayed development of the male (stamen) and female (carpel) fertilizing organ. The flaps of the stigma open not until the style is longer then the filament and protrude clearly above the already overripe anthers (Heeger, 1956).
Self-incompatibility would also lead to allogamy. In valerian, self-incompatibility does probably not exist, because Konon and Novikova (1981) have shown that isolated inflorescences with not manipulated flowers produce germinable inbred seeds. As shown in the material and method chapter, inbreeded plants were also generated in this investigation.

Male sterility can also support cross-pollination, but in the current investigation, no involved plants showing male sterility. Shugaeva (1979) reported from valerian inflorescences, containing normal developted androgynous flowers as well as flowers with male sterility at the same time. The ratio of the two types of flowers should vary from plant to plant and change during the process of flowering. However, the simultaneous appearance of these two types of flowers seems to be an exception. Some less flowers do not lead to the observed OCRs. Therefore, a preference to foreign pollen must exist. Furthermore, genetically based crossing-barriers are be excluded, due to the observed seed development at emasculated valerian flowers, who were pollinated by wind and insects (Konovalova et al., 1978).

If no regulative measures are taken or are naturally available, the generated seeds and descendants, respectively, are predominantly originated by cross-pollination, but a varying proportion will be originated by self-pollination (see in **(ii)**).

(ii) The individual OCR of the combining partners (parents) may be one of the most important factors for the composition with self- and cross-pollinated seeds. The OCR of a pair-combination in this study represented the OCR of the pollen acceptor. The OCRs differed among the pair-combinations PC-A, PC-A_{rzp}, PC-B and PC-C (Figure 2). The OCR may be essentially caused by the pollen acceptor. That is shown at pair-combination PC-B and PC-C which had the same pollen donor (cloned elite 710125) but different OCRs.

However, a good pollen donor does not have to be a good pollen acceptor. This substantiates the different OCRs in reciprocal crosses, shown in pair-combination PC-A and the reciprocal pair-combination PC-A_{rzp} (Table 5, Figure 2). This was also observed in investigations with other plant species, e.g. with *Rhinanthus species L.* by Ducarme and Wesslingh (2013) and with *Borago officinalis* L. by Leach et al. (1990).

In this investigation, we consider the mating system and the composition with self- and cross-pollinated seeds in the harvested seeds, and less which effect or mechanism is behind of the variation of the OCR. Pollen availability and quantity (see also **(iii)**) as well as the pollen quality are presumably the

main causes for the observed variation. Duarte-Silva et al (2011) investigated the pollen viability of *Valeriana scandens* L. and reported that the pollen viability, based on the integrity of vegetative cells, and the performance of in vitro pollen germination was lower, than a pollen stainability test hypothesized. If this is similar in *Valeriana officinalis* L. s.l., selfing and crossing (see in **(i)**) is possible, because of the high pollen quantity produced by the multitude flowers.

(Q2) An established procedure for estimate the OCR in valerian was not available. In the current investigation, such procedure should be developed by using open-pollination crossing-systems and using the AFLP analysis for estimate the OCR. Some aspects, affecting the seed production, were observed and should be discussing **(iii)**. Equally, the AFLP analysis also shows some interesting aspects **(iv)**.

(iii) In order to generate seeds by open pollination, logically, the involved plants must be flowering simultaneous and must produce a sufficient quantity of pollen (see also **(ii)**). The plants of the pair-combinations showed a large overlapping flower period and typical growing. That is important, because intense variation of morphology and ontogenesis can influence the OCR. Carromero and Hamrick (2005) reported higher OCR in taller plants of *Verbascum thapsus* L. and Pathirana (1994) detected higher OCR in seed capsules which were developed in the period of simultaneously flowering of several varieties of sesame (*Sesamum indicum* L.). Both authors concluded that pollinators cause the differences. The kind and abundances of pollinators were not investigated in this investigation, but valerian seems to be a generalist for pollinators (Heeger, 1956). Probably, the more important pollination effect seems to be the wind.

In order to maximize the individual OCR of the mother plant and to prevent other influences, the plants of the combining partners were planted at 0.4 m distance. This is a typical distance in cultivation for root and seed production. The distance between plants can affect the OCR. D'Andrea et al. (2008) determined a 50 % lower OCR in a distance of 1 m to the pollen donor two lettuce species (*Lactuca sativa L.*, *L. serriola L.*). Such detailed investigations are not known for valerian. However, the inter plant distance of 0.4 m of this study appear not to be the limiting factor, considering the development of several shoots per plant and the tallness of the shoots that supports the merging of the shoots.

The distance assume also an important aspect for the pollen-drift and the gene-flow from wild relatives or other varieties into the new variety, and also in other direction. These were investigated in several studies, especially in the context of genetically modified plant material. Van de Wiel and Lotz (2006) gave an overview of the outcrossing situation of maize (*Zea mays L.*), oilseed rape (*Brassica napus L.*), sugar beet (*Beta vulgaris L.*) and potato (*Solanum tuberosum L.*) in the Netherlands, and concluded that 100-200 m isolation distance might be adequate to minimize pollen-drift and gene-flow. Besides the distance, also the wind direction or rather the field orientation to each other can influence the OCR (Hoyle and Cresswell, 2007). To be sure that the calculated valerian OCR were influenced only by the two combining partners, it was ensured that the pair-combinations were placed separate and

not in wind direction to each other. Wild valerian populations in the immediate vicinity were not exist. The OCRs between the two pair-combinations at the same location were not determined due to differing preselective and selective primer of the AFLP assays. Hoyle and Cresswell (2007) determined wind direction and wind speed as the most influencing factors of OCR differences of locations and years. In this investigation, OCR differences between both locations and years were not detectable.

(iv) The AFLP analysis for valerian was adapted and has already been used for determining the relationship between different valerian populations. Heuberger et al. (2012a) noted that different tetraploid valerians showed high genetic similarity and also similar AFLP fingerprints. The similar genetic background of the examined elites results in only few polymorphisms and made the combination finding difficult. For future OCR investigations further primers should be used.

Unlike at diploids, at tetraploids homozygosity is not secured with a present DNA-fragment, because homozygous and heterozygous genotypes can show this band. This must be taken into account when interpreting the results. At the evaluation of pair-combinations PC-A and PC-A_{rzp}, the reciprocally used crossing, not all descendants of the elite 710313 showed the expected own donor DNA-fragment (Figure 1, DNA-fragment 2, position 3, 4 and 10). Thus, this DNA-fragment cannot be homozygous (AAAA). The ratio of present and not present DNA-fragments in the 710313 descendants (without inbreeded descendants like in position 6 and 8) were evaluated. The ratio for phenotypical visibility (+/-) was 5.1/1 (2014) and 4.9/1 (2015). A ratio of 5/1 represents the parents allele-combination aaaa x AAaa (Table 1). The observed ratio was compared and tested for goodness of fit by the chi-square test. In 2014 and 2015 the p-value was 0.95 (χ^2=0.003). Thus, it seems to be a duplex allele-combination in 710313. Initially, only ten inbred descendants (I_1) of each pollen donor were examined for homozygously present DNA-fragments (see in chapter 2.1 Plant material and seed production). However, to avoid a non-detecting of OCR in an acceptor-progeny, at least more than 107 inbreeded plants of the pollen donor must be analysed to find in minimum one plant with an homozygous DNA-fragment (confidence interval = 0.95). The characterization of the allel-combination of the plant-material was not the main aim of this study, but it shows that the determined OCR of 710323 is to be regarded as the lower OCR limit, because some out-crossing became not visible due to the missing DNA-fragment.

The results of the OCR determination of pair-combination PC-A, PC-A_{rzp} and PC-C based just on one DNA-fragment. However, more DNA-fragments do not necessarily increase expressiveness as can be seen in pair-combination PC-B, where two DNA-fragments were available and similar OCRs for the DNA-fragments were observed.

(Q3) The occasion for this investigation arose from the development of a new valerian variety, which should be consisting from the seeds of two open-pollinated parents. The seeds of both parents should be ideally harvested together and handled as mix of seeds. The individual seed yield **(v)**, as well as the thousand-seed weight and the germination capacity **(vi)** of the seed of each parent are traits, who can affecting the composition with self- and cross-pollinated seeds. This can also affect the genetic composition and the field and quality performance of the new variety.

(v) If the seeds of both combining partners are used as seed mix, a high seed yield of both parents is important. However, if the progeny of one combining partner shows outstanding performances, so that it is wanted to fully represent or at least should dominate the new variety, the seed yield of the irrelevant combining partner must be as low as possible. Based on a different number of plants of the both combining partner, could be tried to create an imbalance of pollen availability and fertile flowers. However, the more common, because most practicable variant, will be to plant the two parents in separate rows and the seeds of only one combining partner is harvested.

In the current investigation the individual seed yields of the mother plant rose up in the second year of harvest and showed an average seed yield of 1.9 g per plant. An average seed yield of 5 g per plant, as described by Bomme (2001), was not achieved. This may have been due to the genetic potential of the plant material. The seed yield differences between the combining partners of the pair combination PC-A and PC-B may be also explained by the plant material (Table 6).

(vi) The average germination capacity of 86 % was higher than the recommended minimum germination capacity of 75 % (Aedtner, 2009) and above the germination capacity of commercially available seed lots of valerian (Wahl and Plescher, 2014). Germination capacity usually declines with progressing inbreeding level (Penzkofer et al., 2016). Thus, a high proportion of inbred seeds, indicated by a small OCR, could adversely affect the performance of the new variety. This is supported by the correlation that was observed for germination capacity and OCR, however the validity is limited due to a low number of progenies and high standard deviations (Figure 4).

In contrast to the germination capacity, a higher thousand-seed weight does not necessarily affect the seed quality, but could influence the seed productivity, if a higher thousand-seed weight would lead to a higher seed yield with respect to seed mass. The thousand-seed weight of the combining partners did not differ in this investigation. This is a prerequisite for the mixed seed method to avoid separation and selection during the seed processing.

(vii) The seeds, who are produced by an open pollination mating system and by androgynous fertile combining partners, will be contain always an unavoidably percentage of seed, who are originated by self-pollination. The plants that develop from these self-pollinated seeds often show a lower performance, like a lower vitality and height as well as lesser root and seed yield (Penzkofer et al., 2016, see also **(vi)**). Within the inbreeded plants, found in the current investigation, a distinct inbreeding depression was not observed. Probably the low level of inbreeding is responsible for this. If the crossing were done with combining-partners, who are exhibit a higher level of inbreeding, this would probably be different. Which effect inbred seeds would have on the agronomic and quality performance was not the aim of the current investigation.

Acknowledgements and annotation

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5.2 Figures and Tables

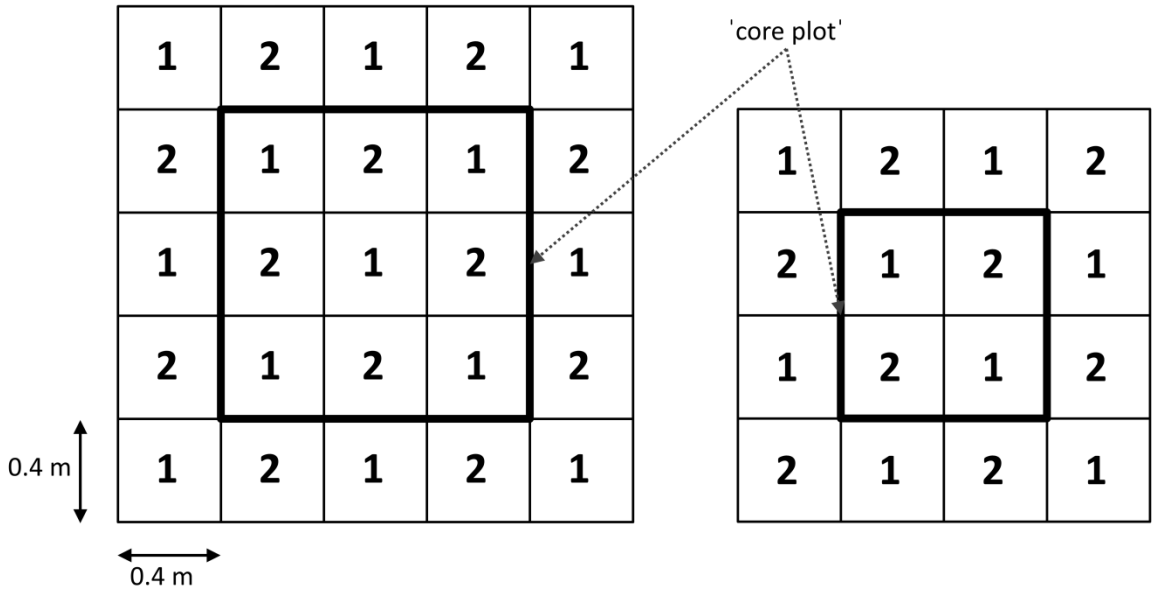

Fig. 1: Planting and crossing schemes of the open-pollinated polycross-systemes (left: Five-rows-five-columns-crossing-scheme, right: Four-Rows-four-columns-crossing-scheme). Combining partner were planted alternately (No. 1 and No. 2). The black and thicker frame in the center of the plant schemes indicates the 'core plot', from which the seeds were harvested and analyzed.

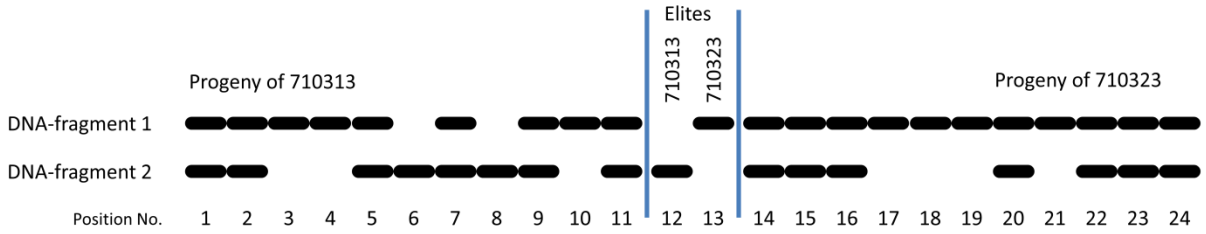

Fig. 2: Schematic example of a DNA-fragment pattern of reciprocal pair-combination PC-A (left: descendants of acceptor mother plant 710313, in case of hybridization DNA-fragment 1 is visible) and PC-A_{rzp} (right: descendants of acceptor mother plant 710323, in case of hybridization DNA-fragment 2 is visible), as well as the DNA-fragment pattern of the acceptor or donor plants (elites, middle) of valerian.

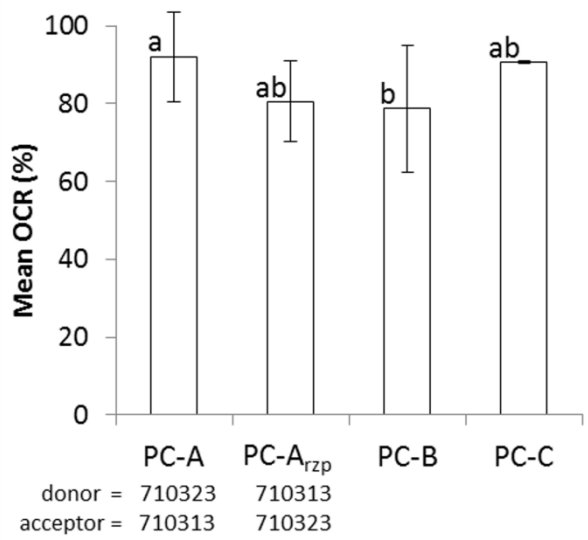

Fig. 3: Effect of the pair combination (PC) on the mean outcrossing rate (OCR) based on n=4 to n=20 individual mother plants of valerian. Kruskal-Wallis Test: $H(3) = 12.48$; $p = 0.005$. Mean values with the same letter are not significantly different ($p < 0.05$, Scheffe-Test). Vertical lines: standard deviation.

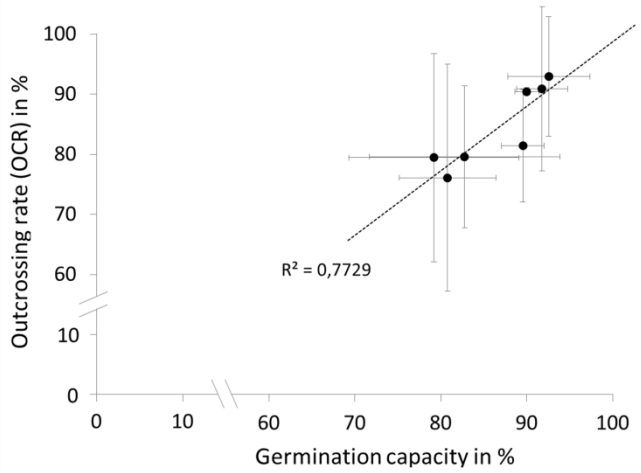

Fig. 4: Correlation of germination capacity and outcrossing rate (OCR) of valerian. R^2 = coefficient of determination of mean; n=4 to n=9 individual mother plants. Vertical and horizontal lines: standard deviation.

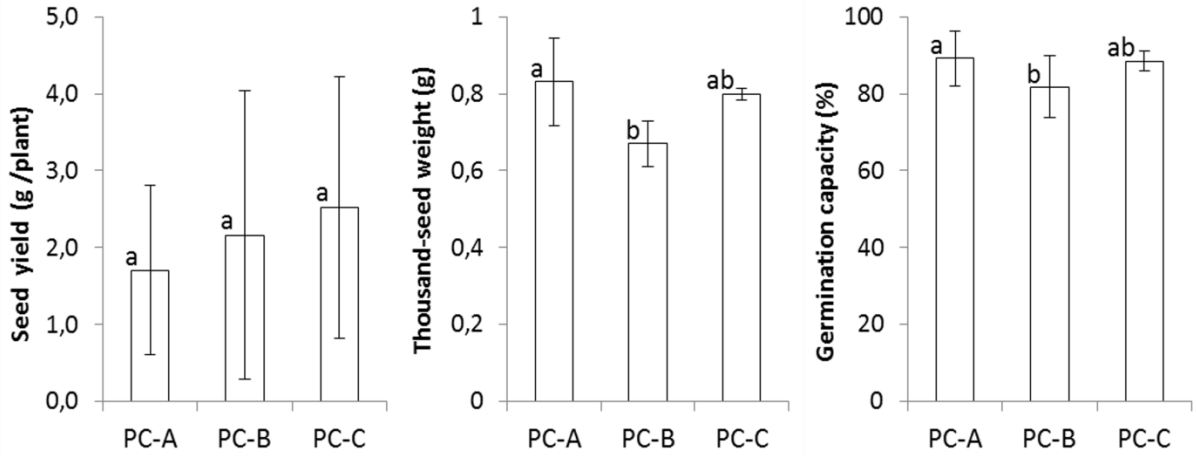

Fig. 5: Effect of pair combinations (PC) of valerian on seed yield, thousand-seed weight and germination capacity; n=4 to n=36 individual mother plants. Kruskal-Wallis Test: Seed yield $H(2) = 1.15$, $p = 0.560$; thousand-seed weight $H(2) = 24.29$, $p < 0.001$; germination capacity $H(2) = 13.33$, $p = 0.001$. Mean values with the same letter are not significantly different ($p < 0.05$, Scheffe-Test). Vertical lines: standard deviation.

Table 1: Combining partners of the pair combinations (PC) of valerian at the two experimental locations (L). Details are described in the text.

Location	**Pair-combination**	**Combining-partner No. 1 Donor**	**Combining-partner No. 2 Acceptor**	**Number of DNA-fragments used as marker**
		Elite No.	Elite No.	
L-1	PC-A	710323	710313	1
	PC-A_{rzp}	710313	710323	1
	PC-B	710125	710217	2
L-2	PC-A	710323	710313	1
	PC-A_{rzp}	710313	710323	1
	PC-C	710125	709933	1

Table 2: Enzyme system, designation and nucleotides sequence of the adapter, preselective and selective primer of the AFLP assays used in the outcrossing study for valerian.

	Enzym system	**Designation**	**Sequence** (5' → 3')
Adapter:	*Eco*RI		CTCGTAGACTGCGTACC CATCTGACGCATGGTTAA
	*Mse*l		GACGATGAGTCCTGAG TACTCAGGACTCAT
First amplification / Preselective primer:	*Eco*RI	primer + 1 (E03)	GACTGCGTACCAATTC G
	*Mse*l	primer + 1 (M02)	GATGAGTCCTGAGTAA C
Second amplification / Selective primer:	*Eco*RI	primer + 3 (E64)	GACTGCGTACCAATTC GAC
		primer + 3 (E76)	GACTGCGTACCAATTC GTC
	*Mse*l	primer + 3 (M49)	GATGAGTCCTGAGTAA CAG
		primer + 3 (M59)	GATGAGTCCTGAGTAA CTA

Table 3: The allele-combination in tetraploid parents (P1, P2) and the distribution in the progeny after crossing and inbreeding, respectively, as well as the phenotypic proportion of the DNA-fragment as a dominant trait.

Allele-combination in the parents		**Distribution of five possible allele-combinations in the progenies by six allele-combination in the gametes.**					**Phenotypical proportion**
P1	**P2**	**AAAA**	**AAAa**	**AAaa**	**Aaaa**	**aaaa**	**+/- [a]**
Crossing							
AAAA	aaaa	0	0	36	0	0	1/0
AAAA	AAAa	18	18	0	0	0	1/0
AAAA	AAaa	6	24	6	0	0	1/0
AAAA	Aaaa	0	18	18	0	0	1/0
aaaa	AAAa	0	0	18	18	0	1/0
aaaa	AAaa	0	0	6	24	6	5/1
aaaa	Aaaa	0	0	0	18	18	1/1
AAAa	AAaa	3	15	15	3	0	1/0
AAAa	Aaaa	0	9	18	9	0	1/0
AAaa	Aaaa	0	3	15	15	3	11/1
Inbreeding [b]							
AAAa		9	18	9	0	0	1/0
AAaa		1	8	18	8	1	35/1
Aaaa		0	0	9	18	9	3/1

[a] + = DNA-fragment exist and is visible onto the electrophoresis-gel image
- = DNA-fragment does not exist and is not visible onto the electrophoresis-gel image

[b] By inbreeding P1 = P2

Table 4: Overview of the number of examined acceptor mother plants, examined plants of acceptor progeny and acceptor progeny plants with donor DNA-fragment, the resulting outcrossing rate (OCR) for three pair-combinations (PC) of valerian, two locations (L) and two trial years.

Pair-combiantion	**Donor**	**Acceptor**	**Location**	**DNA-fragment**	**Trial year**	**Number of examined mother plants**	**Number of examined plants of acceptor progeny**	**Number of acceptor progeny plants with a donor DNA-fragment**	**Outcrossing rate (OCR)**
	Elite No.	Elite No.		No.					[in %]
PC-A	710323	710313							
			L-1 + L-2	1	2014 + 2015	18	239	219	91.6
			L-1	1	2014 + 2015	10	107	96	89.7
				1	2014	5	54	48	88.9
				1	2015	5	53	48	90.6
			L-2	1	2014 + 2015	8	88	83	94.3
				1	2014	4	44	43	97.7
				1	2015	4	44	40	90.9
PC-A_{rzp}	710313	710323							
			L-1 + L-2	1	2014 + 2015	18	239	196	82.0
			L-1	1	2014 + 2015	8	86	68	79.1
				1	2014	4	44	35	79.6
				1	2015	4	42	33	78.6
			L-2	1	2014 + 2015	10	109	89	81.7
				1	2014	5	54	44	81.5
				1	2015	4	44	39	88.6
PC-B	710125	710217							
			L-1	1+2	2014 + 2015	20	208	164	78.9
				1	2014 + 2015	10	104	83	79.8
				1	2014	5	53	42	79.2
				1	2015	5	51	41	80.4
				2	2014 + 2015	10	104	81	77.9
				2	2014	5	53	42	79.2
				2	2015	5	51	39	76.5
				1+2	2014	10	106	84	79.3
				1+2	2015	10	102	80	78.4
PC-C	710125	709933							
			L-2	1	2014		Browsing damage, evaluation not possible		
				1	2015	4	43	39	90.7
Total						60	641	539	84.1

Table 5: Mean outcrossing rate (OCR) of valerian, standard deviation and p-value of independent samples t-test for differences between two locations (L), trial years, DNA-fragments and combining partners of n=8 to n=32 individual mother plants in four pair combinations (PC).

Pair-combiantion	**Donor**	**Acceptor**	**Location**	**DNA-fragment**	**Trial year**	**Test for differences between**	**Mean of individual OCR**	**Standard deviation of indvidual OCR**	**p-value**
	Elite No.	Elite No.		No.			[in %]	[in %]	
PC-A	710323	710313							
			L-1			**location**	90.0	10.9	0.460
			L-2				94.3	12.8	
					2014	**trial year**	92.9	9.9	0.724
					2015		90.9	13.6	
PC-A_{rzp}	710313	710323							
			L-1			**location**	79.1	9.6	0.613
			L-2				81.6	11.4	
					2014	**trial year**	79.6	11.8	0.722
					2015		81.4	9.4	
PC-A, PC-A_{rzp}									
	710323	710313				**combining partner**	91.9 a	11.6	0.004
	710313	710323					80.5 b	10.4	
PC-B	710125	710217	L-1						
					2014	**trial year**	79.5	16.3	0.841
					2015		77.9	17.4	
				1		**marker**	79.6	16.5	0.812
				2			77.8	17.2	
Total									
all (without PC-C, L-2)					2014	**trial year**	84.2	13.8	0.806
					2015		83.2	14.6	

Table 6: Independent samples t-test for differences within two locations, trial years and combining partners for the seed yield, thousand-seed weight and germination capacity of the mother plants. Mean values without a letter are not significantly different ($p < 0.05$, Scheffe-Test).

Pair-combiantion	**Combination partner**	**Location**	**Trial year**	**Mean ± Standard deviation of individual seed yield**		**indvidual thousand- seed weight**		**indvidual germination capacity**	
	Elite No.			[in g]		[in g]		[in %]	
PC-A	710313 + 710323	L-1 + L-2	2014 + 2015	1.70 ± 1.10		0.83 ± 0.23		89.5 ± 22.0	
	710313			2.11 ± 1.08	a	0.85 ± 0.08		92.2 ± 3.9	a
	710323			1.31 ± 1.00	b	0.81 ± 0.14		86.2 ± 8.5	b
	710313 + 710323	L-1		1.78 ± 1.27		0.77 ± 0.09	a	88.4 ± 6.7	
		L-2		1.63 ± 0.94		0.89 ± 0.11	b	89.9 ± 7.8	
	710313	L-1		2.55 ± 1.18	a	0.83 ± 0.04		91.6 ± 3.6	
		L-2		1.56 ± 0.67	b	0.87 ± 0.11		92.9 ± 4.3	
	710323	L-1		0.82 ± 0.50		0.70 ± 0.08	b	84.5 ± 7.8	
		L-2		1.69 ± 1.14		0.90 ± 0.11	a	87.5 ± 9.2	
	710313 + 710323		2014	1.40 ± 0.89		0.76 ± 0.06	b	87.7 ± 9.7	
			2015	2.02 ± 1.23		0.90 ± 0.12	a	90.7 ± 2.9	
	710313		2014	1.70 ± 1.02		0.79 ± 0.03	b	92.6 ± 4.7	
			2015	2.52 ± 1.04		0.91 ± 0.06	a	91.8 ± 3.0	
	710323		2014	1.09 ± 0.66		0.74 ± 0.08	b	82.8 ± 11.1	
			2015	1.52 ± 1.25		0.88 ± 0.16	a	89.6 ± 2.5	
PC-B	710217 + 710125	L-1	2014 + 2015	2.02 ± 1.81		0.67 ± 0.24		82.1 ± 28.3	
	710217			2.94 ± 2.13	a	0.67 ± 0.06		80.0 ± 7.6	
	710125			1.19 ± 0.89	b	0.66 ± 0.06		84.4 ± 8.3	
	710217 + 710125		2014	1.62 ± 1.06		0.64 ± 0.05	b	83.4 ± 8.8	
			2015	2.70 ± 2.40		0.71 ± 0.05	a	80.0 ± 7.1	
	710217		2014	2.18 ± 1.15		0.65 ± 0.06		79.2 ± 9.9	
			2015	3.69 ± 2.74		0.70 ± 0.05		80.8 ± 5.6	
	710125		2014	0.93 ± 0.26		0.62 ± 0.02	b	88.8 ± 2.9	
			2015	1.46 ± 1.27		0.73 ± 0.03	a	78.7 ± 10.4	
PC-C	710125 + 709933	L-2	2015	2.52 ± 1.70		0.80 ± 0.02		88.5 ± 2.5	
	710125			3.67 ± 1.68		0.81 ± 0.02		87.0 ± 2.8	
	709933			1.38 ± 0.78		0.79 ± 0.01		90.0 ± 1.4	
Total	all (without PC-C, L-2, trial year 2014)			1.90 ± 1.42		0.78 ± 0.12		86.9 ± 7.9	

5.3 Supplemented Data (*'supplement table'*)

Table of raw-data. Available also online at https://doi.org/10.1007/s10681-018-2164-9.

Location	Pair-combiantion	Combining partner No.1 (Donor)	Combining partner No.2 (Acceptor)	Marker Number	Trial year	Individual plantnumber	Outcrossing rate (OCR)	Seed yield	thousand grain weight	germination capacity
		Elite No.	Elite No.			Acceptor/mother plant		g	g	%
L-2	PC-A	710323	710313	1	2014	1-8	0.909	1.700	0.793	97
L-2	PC-A	710323	710313	1	2014	1-12	1.000	0.500	0.773	95
L-2	PC-A	710323	710313	1	2014	1-14	1.000	1.300	0.758	97
L-2	PC-A	710323	710313	1	2014	1-18	1.000	1.000	0.747	86
L-2	PC-A	710323	710313	1	2015	1-8	1.000	2.232	0.958	91
L-2	PC-A	710323	710313	1	2015	1-12	1.000	2.325	0.961	88
L-2	PC-A	710323	710313	1	2015	1-14	1.000	2.205	0.992	97
L-2	PC-A	710323	710313	1	2015	1-18	0.636	1.202	0.981	92
L-1	PC-A	710323	710313	1	2014	1-7	0.727	1.700	0.831	84
L-1	PC-A	710323	710313	1	2014	1-9	1.000	1.600	0.816	97
L-1	PC-A	710323	710313	1	2014	1-13	0.818	1.700	0.760	92
L-1	PC-A	710323	710313	1	2014	1-17	1.000	4.200	0.778	92
L-1	PC-A	710323	710313	1	2014	1-19	0.909	1.600	0.818	93
L-1	PC-A	710323	710313	1	2015	1-7	1.000	2.623	0.901	90
L-1	PC-A	710323	710313	1	2015	1-9	0.909	1.262	0.828	94
L-1	PC-A	710323	710313	1	2015	1-13	0.909	4.430	0.863	94
L-1	PC-A	710323	710313	1	2015	1-17	1.000	2.669	0.861	88
L-1	PC-A	710323	710313	1	2015	1-19	0.727	3.709	0.869	92
L-2	PC-A_{rzp}	710313	710323	1	2014	2-7	0.636	0.800	0.793	93
L-2	PC-A_{rzp}	710313	710323	1	2014	2-9	0.818	0.400	0.792	91
L-2	PC-A_{rzp}	710313	710323	1	2014	2-13	0.727	1.100	0.788	63
L-2	PC-A_{rzp}	710313	710323	1	2014	2-17	0.800	2.400	0.844	84
L-2	PC-A_{rzp}	710313	710323	1	2014	2-19	1.000	1.600	0.818	94
L-2	PC-A_{rzp}	710313	710323	1	2015	2-7	1.000	0.892	1.080	92
L-2	PC-A_{rzp}	710313	710323	1	2015	2-9	0.818	3.687	0.903	90
L-2	PC-A_{rzp}	710313	710323	1	2015	2-13	0.727	0.695	1.015	93
L-2	PC-A_{rzp}	710313	710323	1	2015	2-17	0.818	3.340	0.988	90
L-2	PC-A_{rzp}	710313	710323	1	2015	2-19	0.818	1.989	1.001	85
L-1	PC-A_{rzp}	710313	710323	1	2014	2-8	0.909	0.270	0.681	87
L-1	PC-A_{rzp}	710313	710323	1	2014	2-12	0.818	0.870	0.652	89
L-1	PC-A_{rzp}	710313	710323	1	2014	2-14	0.636	0.870	0.680	72
L-1	PC-A_{rzp}	710313	710323	1	2014	2-18	0.818	1.500	0.639	72
L-1	PC-A_{rzp}	710313	710323	1	2015	2-8	0.700	0.830	0.697	91
L-1	PC-A_{rzp}	710313	710323	1	2015	2-12	0.818	0.193	0.647	87
L-1	PC-A_{rzp}	710313	710323	1	2015	2-14	0.900	0.522	0.890	90
L-1	PC-A_{rzp}	710313	710323	1	2015	2-18	0.727	1.541	0.716	88
L-1	PC-B	710125	710217	1	2014	3-7	0.545	3.900	0.679	89
L-1	PC-B	710125	710217	1	2014	3-9	0.800	0.700	0.689	79
L-1	PC-B	710125	710217	1	2014	3-13	1.000	2.300	0.710	89
L-1	PC-B	710125	710217	1	2014	3-17	0.900	1.900	0.568	67
L-1	PC-B	710125	710217	1	2014	3-19	0.727	2.100	0.607	72
L-1	PC-B	710125	710217	2	2014	3-7	0.545	---	---	---
L-1	PC-B	710125	710217	2	2014	3-9	0.800	---	---	---
L-1	PC-B	710125	710217	2	2014	3-13	1.000	---	---	---
L-1	PC-B	710125	710217	2	2014	3-17	0.900	---	---	---
L-1	PC-B	710125	710217	2	2014	3-19	0.727	---	---	---
L-1	PC-B	710125	710217	1	2015	3-7	0.545	5.830	0.731	78
L-1	PC-B	710125	710217	1	2015	3-9	0.909	0.756	0.613	79
L-1	PC-B	710125	710217	1	2015	3-13	0.818	2.090	0.680	74
L-1	PC-B	710125	710217	1	2015	3-17	0.714	2.501	0.745	85
L-1	PC-B	710125	710217	1	2015	3-19	1.000	7.281	0.719	88
L-1	PC-B	710125	710217	2	2015	3-7	0.545	---	---	---
L-1	PC-B	710125	710217	2	2015	3-9	0.909	---	---	---
L-1	PC-B	710125	710217	2	2015	3-13	0.636	---	---	---
L-1	PC-B	710125	710217	2	2015	3-17	0.714	---	---	---
L-1	PC-B	710125	710217	2	2015	3-19	1.000	---	---	---
L-1	PC-B	710217	710125	---	2014	4-8	---	0.700	0.626	92
L-1	PC-B	710217	710125	---	2014	4-12	---	0.900	0.622	89
L-1	PC-B	710217	710125	---	2014	4-14	---	0.800	0.634	89
L-1	PC-B	710217	710125	---	2014	4-18	---	1.300	0.589	85
L-1	PC-B	710217	710125	---	2015	4-8	---	0.095	na	na
L-1	PC-B	710217	710125	---	2015	4-12	---	1.452	0.746	82
L-1	PC-B	710217	710125	---	2015	4-14	---	1.139	0.690	67
L-1	PC-B	710217	710125	---	2015	4-18	---	3.147	0.739	87
L-2	PC-C	710125	709993	1	2015	4-9	0.909	1.934	0.783	89
L-2	PC-C	710125	709993	1	2015	4-17	0.900	0.829	0.796	91
L-2	PC-C	710125	709993	1	2015	4-9	0.909	---	---	---
L-2	PC-C	710125	709993	1	2015	4-17	0.909	---	---	---
L-2	PC-C	709993	710125	---	2015	3-12	---	4.855	0.798	89
L-2	PC-C	709993	710125	---	2015	3-16	---	2.479	0.820	85

6. Discussion and Conclusion

In contrast to other agronomic crops, the use of the genetic potential and the active breeding of medicinal plant species are just at the beginning stages (Pank and Blüthner, 2009). Despite many centuries of traditional use, most medicinal plant species are at best in the transition from wild to cultivated plants with the corresponding natural variability. For economic efficiency during cultivation, it is essential to have efficient, yield-proof, disease-resistant and readily harvestable and processable plants (Franz, 2014). The medicinally used valerian (*Valeriana officinalis* L. s.l.) may be regarded as an exception due to the long breeding tradition (already started in the early 20th century), as well as the large number of developed varieties (Bernath, 1997; Heuberger et al., 2012b). However, the breeding aims of valerian have still changed several times in the past, depending on the actual state of knowledge and the intended purpose. At present, two research and breeding trends for valerian can be observed. The first trend deals with the secondary compounds of the dried valerian root (drug), especially the composition, metabolism and mechanism of action (Felgentreff et al., 2012). The second trend deals with the possibility of increasing the profitability of the field production with more easily processable varieties.

The thesis objectives are strongly associated with a current breeding project, and therefore, the investigations and publications were assigned more to the second trend. This joint research project entitled “Improving the international competitiveness of the German production of medicinal and aromatic plants based on breeding and agro-technological optimizations of chamomile, valerian and lemon balm as model crops” (KAMEL) (Heuberger et al., 2012c; Heuberger and Penzkofer, 2017; Penzkofer and Heuberger, 2018), is related to the alluded second breeding topic.

The species complex of *Valeriana officinalis* L. s.l. forms a multitude of phenotypic and genotypic appearances. Indeed, these complicate allocation to the taxonomic order, but such variable plant material provides an excellent starting point for breeding (Penzkofer and Heuberger, 2019). The plant material used in the current investigations was selected from varieties with breeding background. A long breeding history exists for valerian, and a large number of varieties have been bred and developed (Bomme et al., 1999; Heuberger et al., 2012b). Nevertheless, variability still exists within the varieties (Heuberger et al., 2012a). To avoid adulteration or fuzziness due to variability, clone material from selected and defined single plants (elites) was used for the investigations.

This method has effects on the results validity because the investigated plant material did not represent the entire species complex of *Valeriana officinalis* L. s.l. The results of the relationship of root morphology and contents of secondary compounds, as well as the pollination behavior, are only valid for a limited number of single plants. The single plants that were cloned had been systematically selected to cover fine and coarse-rooted types, as well as types with low and high secondary compound contents. The findings based on these clones provide an initial overview and good tendency for the plant material used in the breeding project. It may be necessary to characterize the variety candidate or the final variety, and for such investigations, appropriate methods and markers are now available.

The thesis objectives depict questions that directly arose from the research and breeding project with the central breeding aim: developing new valerian varieties with a coarser root system and reliable compliance of the root drug with quality regulations of the European Pharmacopoeia (Ph.Eur.9.1, 2017). The first three questions reflect the relationship of the two main breeding characteristics (root morphology and contents of secondary compounds) and their consequences for breeding. The last two questions are concerned with the pollination behavior of valerian and the possible deduced breeding strategies.

6.1 Is there a (negative) relationship between the ingredient content and root diameter, and how do the results influence breeding?

This question arises from the valerian breeding aim to develop varieties with a coarse easy to harvest and process, as well as high contents of secondary compounds in the root system. The root thickness or root diameter, respectively, are the major parameters characterizing a coarser root system. Heuberger et al. (2012c) analyzed three-year data concerning the content of valerenic acid and essential oil, as well as the root morphology of selected single plants (elites). From their data, it seemed that the ingredient contents would decrease with increasing root thickness (Figure 7). If a negative correlation exists for the two characteristics, the breeding aim could never be achieved.

Statistically significant differences in the contents of valerenic acids, essential oil and extractives could not be observed between different root diameter categories (Penzkofer et al., 2014b). The histological investigations further revealed that the relationship between root thickness and the number of oil droplets, as well as the oil droplet density per root cross-section, respectively, was not negative (Penzkofer et al., 2018). Differences were more pronounced between the used plant materials, which were selected from populations with high variability. The populations had been only slightly, if at all, breeder-treated. The populations had been only slightly treated by breeding, if at all. This result was verified and shown by Heuberger et al. (2012a) and Penzkofer and Heuberger (2019), and it was also reflected by the wide range of contents within the same root thickness if a substantial number of data points were available (Figure 7), as well as the variation in contents of the cloned plant material of the same origin (Table 2 in Penzkofer et al. (2014b) and Penzkofer et al. (2018)). The conclusion for breeding is that a potential for developing varieties with both characteristics exists if selection starts from a large gene pool and is carried out exactly and permanently to achieve high performance concerning the breeding traits. In addition, the crossing of plants or lines that are strong in only one characteristic is likely to result in progenies with combined characteristics (Heuberger et al., 2012c; Heuberger and Penzkofer, 2017; Penzkofer and Heuberger, 2018).

6.2 In which parts of the root system are the secondary compounds localized, and how do the results influence the production of the dried root drugs and breeding?

Ingredient localization was studied at different levels, the individual root system compartments (roots and rhizome) and the cellular level. The localization of valerenic acid in the valerian root system was not described in the literature, since the pharmaceutical relevance of the compound was identified comparatively late in the course of the drug use history (Penzkofer and Heuberger, 2019). Descriptions about the localization of essential oil are more numerous and date back partly to the early 20th century (Tschirch and Oesterle, 1900; Fridvalszky, 1957; Holzner-Lendbrandl, 1963; Kutschera, 1992). The greater part of information about essential oil is available for the roots and less for the rhizome. However, the rhizome is not free of secondary compounds (Eisenhuth, 1956; Argyropoulos et al., 2013), but as now known, the ingredient contents are substantially reduced in contrast to the lateral and adventitious roots (Penzkofer et al., 2014b).

The common determination of the secondary compound contents of the valerian root drug is related to the root mass and is normally applied to a mixture of all root system compartments. Therefore, the proportion of each compartment can influence the absolute ingredient contents. The rhizomes captured the lowest proportion of the root mass and contained the lowest contents of secondary compounds. Quite different results have been obtained for adventitious roots. The adventitious roots have the highest proportion of root mass and contain clearly higher contents of secondary compounds (Penzkofer et al., 2014b). Therefore, the low secondary compound contents of the rhizomes may be compensated by the low mass proportion within the total dried root drug.

Caution is required when inbred lines are evaluated. Some valerian inbred lines show strongly reduced growth and root yield caused by high inbreeding depression (Penzkofer and Heuberger, 2019). The root-rhizome-ratio has been observed to be shifted toward the rhizome. This altered ratio may have sometimes contributed to the observed lower ingredient contents in the third inbred generation.

The current investigations in this thesis regarding essential oil showed that 43 % of the observed essential oil droplets were localized in the cell layers near the root surface (Penzkofer et al., 2018). These results are in good agreement with early studies (Violon et al., 1983; Szentpetery et al., 1966); however, it has also been shown that 57 % of the observed essential oil droplets can be found in the whole root cortex.

Penzkofer et al. (2018) discuss their results with respect to the production of the root drug and breeding. The high concentration of essential oil in cells near the root surface would explain why during intense and long washing of fresh valerian roots, which damages the root surface, losses of secondary compounds are often observed (Heindl and Hoppe, 2010). Therefore, it can be assumed that the more essential oil is stored in the parenchymal regions, far from the root surface (inner parenchyma), the lower should be the losses during processing, and the higher will be the processing stability of the roots. This topic was of minor relevance in past breeding because increasing the essential oil content has, thus far,

been a more important target (Bernath, 1997). Penzkofer et al. (2018) showed that different cloned single plants have different essential oil distributions. Future breeding approaches should be conducted to analyze the essential oil distribution within the root sections to select appropriate genotypes. Such appropriate genotypes are characterized by a steadily increasing oil droplet density curve. The roots of such plants will have a higher portion of the essential oil located in the inner parenchyma, which may result in better processing and storage stability. Our imaging and mapping method, in combination with the calculated density curves, can serve as a selection tool for identifying suitable plants.

The comparison of different harvest depths, specified as horizons, provides further information about the reliability of the production process. In deeper horizons the roots are thinner and the likelihood that they will be lost during the harvesting process are greater. The root slices in the deeper horizons showed a higher oil droplet density in fact, but - due to the smaller root diameters in these horizons - contained the fewest oil droplets per slice. Thus, the harvesting of root parts in deeper soil layers does not have to be exhaustive to obtain overall high essential oil contents in the root drug. In addition, restricting the root harvest horizon to the upper soil layer may be economically advantageous, especially if 96 % of the root mass is located in the topmost 10 cm of the ground, as previously described (Neumaier, 2017).

The low-volatile sesquiterpene acids (valerenic acid and acetoxyvalerenic acid) are components of distilled essential oil (Reichling et al., 1994), which has also been observed during an analytical investigation of the localization of valerenic acid within the valerian root system ((Penzkofer et al., 2014b) data not published). However, the distribution of sesquiterpene acids directly from the histological results of the oil droplets could not be determined.

Initially, evidence for the appearance of sesquiterpene acids within the oil droplet in the root was missing. The use of matrix-assisted laser desorption ionization (MALDI) as a mass spectrometry imaging (IMS) technique could provide relief. For example, Miyoshi et al. (2018) analyzed the asparaptine distribution at three positions of the asparagus stalk. Moreover, the application of various vibrational spectroscopy methods for visualizing secondary metabolites in different plant tissues has been previously described for essential oil components in fennel, chamomile and curcuma (Baranska et al., 2004). Similar techniques could also be applied for the sesquiterpene acids in valerian.

Furthermore, the contents of valerenic acid were clearly lower in the distilled essential oil, as in the corresponding root drug. This finding raises the question of whether the sesquiterpene acids were located within the oil droplet at all. The lower contents could be explained by the low volatility of valerenic acids, what means that the valerenic acid could merely have been entrained during the distilling process and merged with the essential oil. Unfortunately, the distillation hydrolate was not investigated with respect to valerenic acid.

6.3 What is the proportion of outcrossing under conditions of open pollination in the breeding material?

High outcrossing rates are needed to develop hybrids in valerian, since an efficient hybrid mechanism is lacking (Penzkofer and Heuberger, 2019). The average proportion of outcrossing under conditions of open pollination was 84 %. The observed outcrossing rates ranged from 76.5 % to 97.7 %, and in some mother plants up to 100 % (Penzkofer et al., 2018). The current results confirmed the previous assumption that *Valeriana officinalis* L. is preferentially cross-pollinated.

High outcrossing is required if two characteristics of two different geno- or phenotypes should be combined by using an open pollination mating system. Open pollination is currently the commonly used method in valerian seed production. To ensure a similar fertilization probability for all pollen grains, attention should be paid to the following aspects:

- the inflorescence and flower development, as well as the flowering time, should be similar for each combining partner,
- the number of individuals of each combining partners is balanced in the seed propagation,
- the arrangement of the combining partners does not hinder the cross-pollination.

Penzkofer et al. (2018) emphasized that the plants of the investigated pair-combinations should show a large overlapping flower period, as well as similar and homogeneous growth habits. Both, morphology and ontogenesis can influence the outcrossing rate. Carromero and Hamrick (2005) reported higher outcrossing rates in taller plants of *Verbascum thapsus* L., and Pathirana (1994) detected higher outcrossing rates in seed capsules that were developed in the period of simultaneous flowering for several varieties of sesame (*Sesamum indicum* L.).

To maximize the individual outcrossing rates (OCR) of the mother plant and to prevent other influences, the plants of the combining partners were planted at a distance of 0.4 m (Penzkofer et al., 2018), which is a typical distance in cultivation for root and seed production. The distance between plants can affect the OCR. D'Andrea et al. (2008) determined a 50 % lower OCR using a distance of 1 m to the pollen donor at two lettuce species (*Lactuca sativa L.*, *L. serriola L.*). Such detailed investigations are not known for valerian. However, in comparison to lettuce, the inter plant distance of 0.4 m in this study likely did not limit outcrossing, considering the development of several shoots per plant, the tallness of the shoots and the expansive inflorescences supporting the merging of the shoots and inflorescences of the combining partners.

The distance also plays an important role in the pollen-drift and gene-flow from wild relatives or other varieties into the new variety, and vice versa. The necessary distances for isolation were investigated in several studies, especially in the context of genetically modified plant material. Van de Wiel and Lotz (2006) provided an overview of the outcrossing situation of maize (*Zea mays L.*), oilseed rape (*Brassica napus L.*), sugar beet (*Beta vulgaris L.*) and potato (*Solanum tuberosum L.*) in the Netherlands, and they concluded that an isolation distance of 100-200 m might be adequate to minimize pollen drift and gene flow. In addition to the distance, the wind direction can also influence the OCR

(Hoyle and Cresswell, 2007). To certify that the calculated valerian OCRs in this study were influenced only by the two combining partners, it was ensured that the pair-combinations were planted at a minimum distance of 150 m. Wild valerian populations in the immediate vicinity did not exist. Hoyle and Cresswell (2007), using maize, oilseed rape and sugar beet, determined the wind direction and wind speed as the most influential factors in the differences in OCRs for locations and years. In this investigation, OCR did not differ between either locations or years.

6.4 What is the probability of developing hybrid- or synthetic-like valerian varieties?

There are some benefits of valerian hybrid varieties, such as performance enhancement, reproducibility, and homogeneity. As a result, the valerian breeding project has accelerated such varieties (Heuberger et al., 2012c; Heuberger and Penzkofer, 2017; Penzkofer and Heuberger, 2018).

In predominantly cross-pollinated (allogamic) plant species, valerian can now be allocated to them, and the highest benefit of a (F_1-)hybrid variety breeding strategy lies in the greater homogeneity within the variety (Becker, 2011). This outcome would be an improvement compared with the currently commonly used population varieties. However, too little data are still available to provide verified statements concerning the homogeneity of the hybrids (Penzkofer and Heuberger, 2019).

A further benefit can be observed in their reproducibility, which presupposes the amount of variety starting plant material and can be preserved for future seed production. Classically, this is done using homogeneous inbred lines or clones of the starting plant material. Methods for inbreeding have been developed (Vömel and Hölzl, 1979; Konon and Novikova, 1981; Heuberger et al., 2012c; Heuberger and Penzkofer, 2017; Penzkofer and Heuberger, 2018; Penzkofer and Heuberger, 2019). Due to the tetraploid plant material, it must be assumed that in the third generation of inbreeding, no complete homozygosity has yet been achieved. A level of 97 % homozygosity is statistically reached after the 21st generation starting from a duplex genotype or after the 19th generation starting from a triplex genotype (Penzkofer and Heuberger, 2019). Considering the high inbreeding depression. occurring from the third inbred generation, and because complete homozygosity cannot reached by classic inbreeding, preservation of the variety starting material is not possible using inbred lines and is also not expedient. A further method to preserve the variety starting plant material is vegetative propagation (the production of clones). Methods for the production of clone plants have also been developed and are comparatively easy to apply (Penzkofer et al., 2018).

The third benefit, which is often demonstrated for hybrid varieties, is the performance enhancement (heterosis). Penzkofer and Heuberger (2019) described such a performance enhancement regarding some performance characteristics in inbred line crossings (F_1) of valerian. In particular, vitality-dependent characteristics (e.g., dried root yield, time until full ground coverage) showed heterosis. Characteristics that were already present in both combined inbred lines (thickness of adventitious roots) showed less heterosis. The selection of suitable combining partners must be ascribed great importance.

The production of F_1-hybrid varieties is usually performed by crossing well-defined combining partners. However, manual crossings of valerian are not truly practical to produce a sufficient amount of crossing seeds and the use of gametocides does not seem to be acceptable for medicinal plants (Penzkofer et al., 2018). Therefore, a system or method for supporting the crossing must be identified and applied.

A male sterility system has been previously described in valerian and can support cross-pollination, but in the current investigation, as well in the breeding plant material, no male-sterile plants were observed (Shugaeva, 1979; Heuberger et al., 2012c).

Self-incompatibility would also lead to allogamy, but self-incompatibility probably does not exist, at least for tetraploid valerian. Konon and Novikova (1981) have shown that isolated inflorescences with unmanipulated flowers produce inbred seeds that are able to germinate. Inbred plant material was also generated in the investigations and during the practical part of the related breeding program (Penzkofer et al., 2018; Penzkofer and Heuberger, 2019).

Genetic incompatibility, which leads to nonviable seeds and has been described, e.g., by Burkart-Waco et al. (2012) for *Arabidopsis thaliana*, cannot excluded in valerian. However, this phenomenon was not investigated thus far. Depending on the consideration, the protandric flowers of valerian can also be considered as a kind genetic regulation (Heeger, 1956). However, due to the multitude of single flowers with different development stages within the same inflorescence, this ontogenetic aspect cannot be used for the regulation of cross pollination.

The described crossing supporting systems cannot used for the development of classic F_1-hybrid varieties, for the present. Therefore, other concepts have been developed and postulated by Penzkofer et al. (2016). The approach is to use the tendency for natural cross-pollination, in combination with highly inbred parental hybrid-components, and allow them to produce hybrid seeds under open pollination conditions. If both combining partners are similarly fertile, the seeds formed are a mixture of seeds originating from reciprocal crossings and inbreeding. Ideally, the inbred seeds do not exist or are quantitatively negligible. The determination of a maternal effect is difficult to achieve because the inflorescences become quite tangled with each other, and the seeds can usually not be attributed to the respective combining partner. Otherwise, an exact knowledge of different seed parameters (e.g., weight, size, shape) of the combining partners must be available to develop seed separation techniques. Therefore, the harvested seed lot will certainly contain a fraction of inbred seeds. This fraction must be maintained in tolerable amounts. Inbred seeds usually show lower vitality and a reduced seedling growth rate, and they will be either suppressed or compensated by the hybrid plants in the established field crop (Bernath, 1997; Penzkofer and Heuberger, 2019). It would also possible be to detect inbred young plants during cultivation, for example, by genome analysis, and eliminate them before planting (Penzkofer et al., 2018).

Within the related breeding programs, crossings of highly inbred single plants with outstanding performance were detected (Heuberger et al., 2012c; Heuberger and Penzkofer, 2017; Penzkofer and

Heuberger, 2018). Therefore, it makes sense to clone the two parents and to generate the F_1 offspring based on these clones and by open pollination. The initiation of this concept is similar to the process used for synthetic varieties (Acquaah, 2012). The difference is the number of components, which are incorporated into the basic seed production (F_1 or Syn_1). For synthetic varieties, high outcrossing rates are usually also necessary, and the produced seeds are also probably a mix of crossing and inbred seeds. The consequent step would be the generation of F_2 from F_1, comparable to the synthetic stages (Syn_1, Syn_2, etc.).

Especially considering the use of clone plants as components, the production of F_2 populations is a common method in breeding to obtain a larger amount of seeds at a low cost. By using highly inbred components, the genetically variability would be limited and channeled.

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