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The Influence of Gibberellic Acid on Essential Oil Production in Thyme (*Thymus vulgaris* L., Lamiaceae)

Master Thesis

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Abstract

The Influence of Gibberellic Acid on Essential Oil Production in Thyme (*Thymus vulgaris* L., Lamiaceae)

Keywords: Gibberellic acid, essential oil, Lamiaceae, *Thymus vulgaris*, glandular hairs.

Essential oils (EOs), a class of secondary metabolites, which are the most important raw materials of the fragrance and aroma industry.The essential oil production depends not only on genetic factors but also on environmental factors. Growth regulators can influence the growth and essential oil production. Thyme (*Thymus vulgaris*L., Lamiaceae) is one of the most important medicinal and aromatic plants which is used almost everywhere in the world with antioxidant, antimicrobial, antiseptic and several medicinal properties such as antimutagenic activity. The active compounds of thyme composed mainly of the monoterpenes (part of the EO like thymol and carvacrol), phenolic diterpenoids (rosmarinic acid derivatives) and flavonoids (flavones). The bioactive essential oil in *Thymus vulgaris* and in many other species of the Lamiaceae family is produced in specialized epidermal glandular hairs spread over the aerial vegetative and reproductive organs and is stored in the subcuticular space of the essential glands which are responsible for their specific flavor. As a plant growth regulator gibberellic acid (GA_3) regulates diverse activities in plants and contribute to the promotion of several processes including seed germination, shoot elongation, flowering, cell division and cell elongation, dormancy, sex expression, enzyme induction and leaf and fruit senescence.In this experiment, we analyzed the effects of the external foliar application of three different concentrations of gibberellic acid (GA_3) on two thyme cultivars (“Deutscher Winter” and “Varico 2”). Our main focus in this research was on how GA_3 is increasing the essential oil content in thyme. But interestingly, we found that when applied highest concentration (5ppm) of GA_3 a decrease of the essential oil content for both cultivars (“Deutscher Winter” and “Varico 2”). Neither the number nor the sizes of the oil glands were decreased by GA_3.

Kurzfassung

Der Einfluss von Gibberellinsäure auf die Produktion von ätherischem Öl in Thymian (*Thymus vulgaris* L., Lamiaceae)

Schlagworte: Gibberellinsäure, ätherisches Öl, Lamiaceae, Thymus vulgaris, Drüsenschuppen

Ätherische Öle (EO) sind pflanzliche Sekundärstoffe, die zu den wichtigsten Rohmaterialien in der Parfüm- und Aromenindustrie zählen. Die Produktion von ätherischem Öl in der Pflanze ist nicht nur abhängig von der Genetik der Pflanze, sondern auch von Umwelteinflüssen. Wachstumsregulatoren können sowohl das Wachstum der Pflanze als auch die Bildung an ätherischem Öl beeinflussen.Der Thymian (*Thymus vulgaris* L., Lamiaceae) ist eine der wichtigsten Arznei- und Gewürzpflanzen aufgrund seiner antioxidativen, antimikrobiellen, antiseptischen und einigen medizinischen Aktivitäten, wie etwa antimutagener Aktivität verwendet. Die für die Aktivität verantwortlichen Inhaltsstoffe sind dabei hauptsächlich Monoterpene (Teil des ätherischen Öles wie etwa Thymol und Carvacrol), phenolischeDiterpene (Rosmarinsäue) und Flavonoide (Flavone). Das bioaktive ätherische Öl wird in Thymian (aber auch in anderen Arten der Lippenblütler) in spezialisierten epidermalen Öldrüsen gebildet, die über die oberirdischen Laub- und Blütenblätter verteilt sind, und wird in einem subkutikulären Raum dieser Öldrüsen gespeichert. Der Wachstumsregulator Gibberellinsäure reguliert diverse Vorgänge in der Pflanze wie etwa der Keimung, Sprossverlängerung, Blüte, Zellteilung und –wachstum, Dormanz, Geschlechtsausbildung, Enzyminduktion und Blatt- und Fruchtseneszenz.In diesem Experiment untersuchten wir den Effekt einer externen Blattapplikation von drei Konzentrationen von Gibberellinsäure (GA_3) bei zwei Thymiansorten („Deutscher Winter“ und „Varico 2“) mit der Frage, ob GA_3 den Ölgehalt in Thymian erhöht. Interessanterweise führte aber die höchste GA_3-Konzentration (5 ppm) zu einer Verringerung des Ölgehaltes in beiden Sorten. Auf die Anzahl und Größe der Öldrüsen gab es keinen Einfluss.

1. Introduction

1.1. Essential oils

Essential oils (EOs), a class of secondary metabolites, are broadly distributed in plant kingdom. Approximately 90% of global EO production is consumed by the flavor and fragrance industries, which is mostly in the form cosmetics, perfumes, soft drinks and food (Lubbe and Verpoorte, 2011). Essential oils are the most important raw materials of the fragrance and aroma industry. They are also used in the food and pharmaceutical industries due to their therapeutic, antimicrobial and antioxidant activities. Nevertheless, they have biological activities that make them able to be used as herbicides, pesticides and anticancer compounds (Mahmoud and Croteau 2002; Abrahim et al., 2003; Burfield and Reekie2005). It has been observed that essential oils have a wide spectrum of activity against bacteria, fungi, pathogens and yeasts (Bagamboula et al., 2004; Hulin et al., 1998).The essential oils are related to plant defense and pollinator attraction among other ecological functions. As other secondary metabolites groups, these compounds play an important role in the plant's fitness under environmental variation. For this reason, a common problem that occurs in aromatic plants cultivation is the quantitative and qualitative variation in response to the environment (Taiz and Zeiger 2004).

1.2. *Thymus vulgaris*

Thyme (*Thymus vulgaris* L.) belongs to the Lamiaceae family and is a pleasant smelling perennial shrub, which grows in several regions in the world (Davis, 1982). Thyme is native to Western Mediterranean region and Southern Italy, where some species form a special type of bushy vegetation not more than 50 cm high, well adapted to hot and dry summer weather. It is a well-known aromatic plant used for medicinal and spice purposes almost everywhere in the world. The beneficial effects

of thyme are well known from ancient times and consumption of its extract is recommended all over the world. In ancient times, it was used by the Egyptians asunguents for embalming and then by the Greeks as incense in their temples and by the Romans in cooking, as a source of honey and therapeutic purposes (Barros et al., 2010).

Essential oils extracted from fresh leaves and flowers can be used as aroma additives in foods, pharmaceuticals and cosmetics (Simon et al., 1999 and Senatore, 1996). Thyme also possesses various beneficial effects as antiseptic, carminative, antimicrobial and anti-oxidative properties (Baranauskiene et al, 2003). Recently, thyme has become one of the most important medicinal plants used as a natural additive in poultry and livestock feeding industries. Such studies showed that thyme plant could be considered as an alternative natural growth promoter for poultry instead of antibiotics (Abu-darwish and Abu-dieyeh 2009).

Figure 1: *Thymus vulgaris* L., thyme (picture from Köhlers Medicinal Plants, 1887, downloaded from

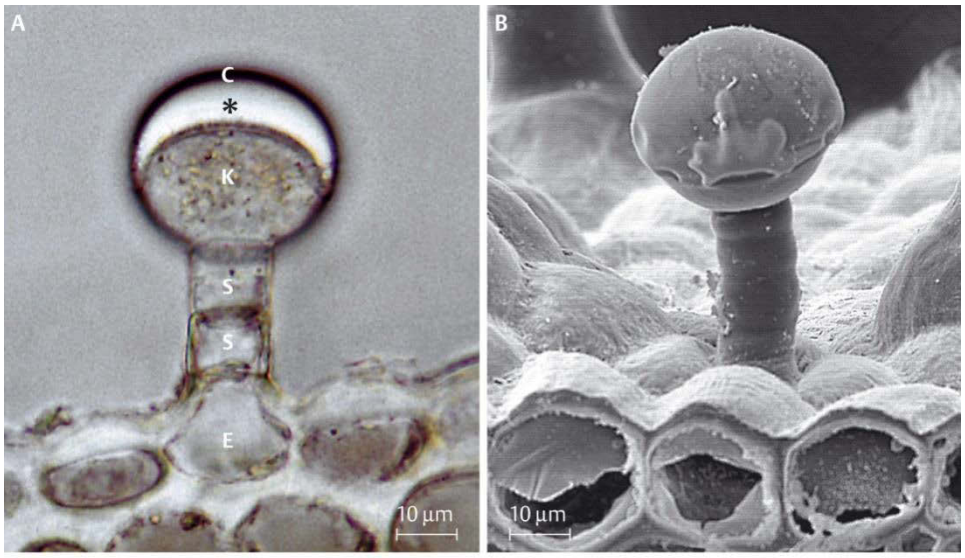
https://commons.wikimedia.org/wiki/Alphabetical_name_index_of_K%C3%B6hler%27s_Medizinal-Pflanzen_-_english?uselang=de#/media/File:Thymus_vulgaris_-_K%C3%B6hler%E2%80%93s_Medizinal-Pflanzen-271.jpg, last time assessed: 25.10.2016)

Schwarz et al. (1996) reported that the main components of *Thymus vulgaris* L. were thymol, carvacrol, and *para*-cymene. Thymol, which is the principal constituents of thyme oil have been reported to act as antioxidant, antimicrobial agent, antifungal agent treatment for respiratory tract diseases, wound healing, a stomachic

carminative, diuretic, urinary disinfectant and vermifuge. Thymol showed strong effect on common respiratory tract (Inouye et al., 2001). A remarkable example is the inhibitory activity of thymol and carvacrol against *Escherichia coli* 0157:H7 that was treated in beef meet and the fact that they act effectively against pathogen in high temperatures (Solomakos et al., 2008; Burt and Reinders, 2003). It is the worth mentioning that carvacrol showed antibacterial activity against *Salmonella* in fish pieces maintained at 4ºC (Bagamboula et al., 2004; Hulin et al., 1998) and antitoxic and anti-pathogenic properties towards *Bacillus cereus* on rice (Ultee et al., 2000).

The bioactive essential oil components in *Thymus vulgaris* species of the Lamiaceae family are produced in glandular hairs spread over the aerial vegetative and reproductive organs and stored in the subcuticular space of the epidermal essential oil glands (Novak *et al*., 2006). It has been observed that there are two types of glandular hairs in the species of Lamiaceae which are differentiated to each other regarding their morphological structure, their secretion mode and their secretion time (Werker, 1993). With reference to their morphological structure they are separated into capitates and peltate glandular hairs (Schmiderer*et al*., 2008). Two types of capitatetrichomes have been observed, trichomes that consist of one basal secretory epidermis cell, one stalk cell and a cylindrical head of two broad cells (capitate type I) and trichomes that consist of a stalk cell, a neck cell and a single avoid head cell (capitates type II). The later type is usually based along the leaf veins on the abaxial side of the leaves. Peltate or scale-like trichomes consist of one basal secretory epidermis cell, one stalk cell and a cylindrical head of 6-14 perimetric cells or sometimes of only four cells (Bisio et al., 1999; Werker, 1993). Concerning the secretion mode and time, the glandular hairs can be distinguished in two types, the short-term glandular hairs related to capitate glandular hairs whose secretory materials are extruded to the outside soon after their production and the secretion is soon terminated before the hairs being touched acting as repellents against herbivores and pathogens. From the other side, there are the long-term glandular hairs related to peltate glandular hairs whose secretory materials are extruded from the basal secretory epidermis cells into a subcuticular space with an appearance

either as a lipophilic substance or as an emulsion, implying regarding to the latter the presence of more than one type of chemical compound. The peltatetrichomes are long-term due to the fact that they continue to be accumulated during the growth of the organ unless they are touched where the cuticle will rupture(Werker 1993).



- Figure 2: Microscopic pictures of an essential oil gland (C…cuticula, K…secretory cell, S…stalk cell, E…basal cell), G. Wanner (2005)

1.3. Gibberellic acid (GA_3)

Plant growth regulators (PGRs) can influence growth and essential oil production. Endogenous level as well exogenous application could affect essential oil production (Prins et al., 2010). The gibberellins, important hormones of vascular plants, fungi and bacteria, contain more than 125 compounds with similar structure (MacMillan, 2001; Rademacher, 2000). Gibberellins regulate diverse activities in plants and contribute to the promotion of several processes including seed germination, shoot elongation, flowering, cell division and cell elongation, dormancy, sex expression, enzyme induction and leaf and fruit senescence. They are also associated with

juvenile to adult transition processes, promote fructification and play an important role on seed germination by activation of embryo vegetative growth and mobilization of energetic reserves from endosperm (Taiz and Zeiger 2004; Spartz and Gray 2008). In comparison with various promotional hormones, gibberellic acid (GA_3) proved as the most effective plant growth regulator in stimulating growth, essential oil content, leaf area and branching (Weiss, 1997).

Growth and essential oil yield of *Menthapiperita* were improved by the application of polyamines (Youssef *et* al., 2002). Silva *et* al., (2005) reported that auxin and cytokinin increased some components of the lemon balm oil. Povh and Ono (2007) showed that application of gibberellic acid influenced the chemical composition of *Salvia* oil. A report revealed that sodium salt of NAA (1-naphthaleneacetic acid, a synthetic auxin) and IAA (indole-3-acetic acid, the most common naturally occurring auxin) increased the essential oil of *Menthapiperita* (Koseva- kovacheva and Staev 1978).The essential oil content increased with increasing levels of active GA_3 in the plant. An increase of monoterpenes by foliar application of active GA_3 is already a well-known procedure for some economically important essential oil crops like *Pelargonium sp., Ocimum sanctum, Ocimumbasilicum, Cymbopogonjawarancusa and Artemisia annua* (reviewed by Sangwan et al., 2001).

The content of the essential oil and its composition in thyme depends on different factors, cultivation conditions such as climate, geographic origin, harvesting time and use of fertilizers. Our main focus of this study is to demonstrate that the effect of gibberellic acid on essential oil glands and essential oil of *Thymus vulgaris*.

2. Materials and Methods

This study/ research wascarried out at the greenhouse of the “Institute of Animal Nutrition and Functional Plant Compound”, at the University of Veterinary Medicine in Vienna during 2014 and 2015. The aim of this study was to elucidate the influence of Gibberellic acid on the production of essential oil in *Thymus vulgaris*. The details of the materials and methods used in the study are described in below:

2.1. Plant material used

For this research, two cultivars, “Varico 2” and “Deutscher Winter” were clonally propagated in September 2014. Plants were cultivated in a glasshouse of the “University of Veterinary Medicine”, Vienna under controlled environment. The samples were harvested in March 2015.

2.2. Gibberellic acid treatment

Gibberellic acid (synonyms: GA_3, Gibberellin A_3, C19H22O6, MW: 346.37, CAS-Nr. 77-06-5) was bought from Merck (Darmstadt, Germany). Three Gibberellic acid (GA_3) treatments (control, 1ppm and 5ppm) were applied on each plant. GA_3 was sprayed once per week for three weeks until drops onthe leaves were observed.

2.3. Sampling and drying

The stems of four replications were cut one week after the last treatment. Then the stems were pressed in a herbarium press in order dry them and to get flat samples for analysis. The herbarium press with the samples was kept in a dryer (Memmert, Modell 800, Schwabach, Germany), at 30°C for 5 days. Afterwards, dried samples were kept at room temperature in the herbarium until analysis.

Figure 3: Thyme (*Thymus vulgaris* L.) (http://www.herblywonderful.com/herbs/thyme-english.jpg, last time assessed: 25.10.2016)

2.4. Experimental design

The experiment was consisted of the three factors listed below:

Factor A. (Cultivars)

1. Varico 2 - (V)
2. German winter - (W)

Factor B. Gibberellic acid (GA_3)

1. No treatment (Control) -0ppm
2. Treatment 1 – 1ppm
3. Treatment 2 – 5ppm

Factor C. Insertion level of the leaf (3 leaf ages)

1. Young– 1st node
2. Medium- 3rd node
3. Old - 5th node

The experiment was laid out in a random block design with 4 replications.

2.5. Statistical analysis

All recorded data were prepared for further analysis in a Microsoft office excel sheet. Then the recorded data were checked for outliers, typing errors and afterwards were analyzed statistically using 3-factorial analysis of variance (ANOVA) technique with the factors cultivar, age and GA_3 concentration. The statistically significant mean difference at $P<0.05$ was calculated by theTukey B test. All statistical data were analysed by IBM SPSS software version 20 (IBM, Vienna, Austria).

2.6. Analysis of plant-materials

2.6.1. Leaf weight

Three leaves per treatment and replication were selected for measuring the dry weight of sample by using an electronic high accuracy balance (Sartorius).

2.6.2. Leaf area, circumference, length and width

Afterwards, the samples were placed into A4 paper sheets and scanned by a scanner (Aficio MP C300, RICOH, Vienna, Austria) with a resolution of 600dpi with the aim to measure parameters of leaf shape such as leaf area, length of the leaf width and circumference of the leaf. All samples were analyzed using Irfanview 4.38 and Image J 1.50. Leaf area, leaf length, leaf width and leaf circumference were counted by mm^2.

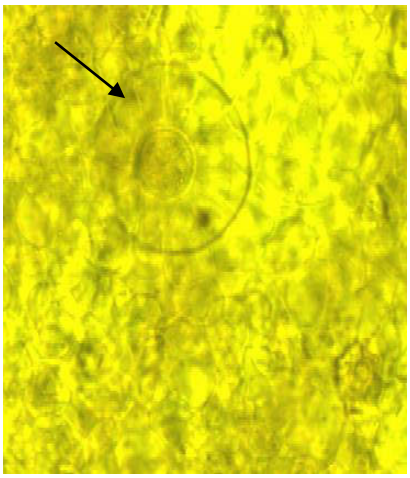
2.7. Counting and measuring gland sizes

To measure gland sizes were used chloral hydrate solution to brighten the sample. We used chloral hydrate, purum, crystallized ≥ 98.0% (T), ALDRICH (Wien, Austria).

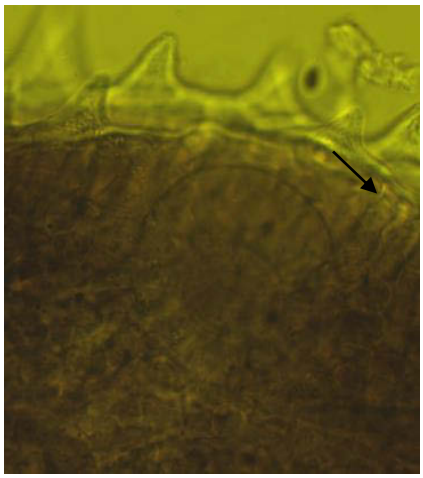
Solution: 160g: Chloral hydrate; 100ml: Aqua Dest. 50ml: Glycerin

Dry samples were put on a glass slide with chloral hydrate solution and covered with cover slip. Afterwards, all samples were heated carefully with on oven at 100°c temperature. Tomeasure gland sizes pictures of samples were taken using a research microscope PROVIS AX70 together with the HV-C20 (color camera) (Olympus, Vienna, Austria).

In the microscope, always the lower side of the leaves was observed and the oil glands were counted visually. The photos were transferred with a micros SD card reader to the computer and via the scientific image analysis software ImageJ and 2 mm long scale (C. REICHERT, company in Wien) was used to measure gland size. ImageJ measures the area in pixels. Gland size was counted by µm². The area of 3 oil glands per leaf was measured.



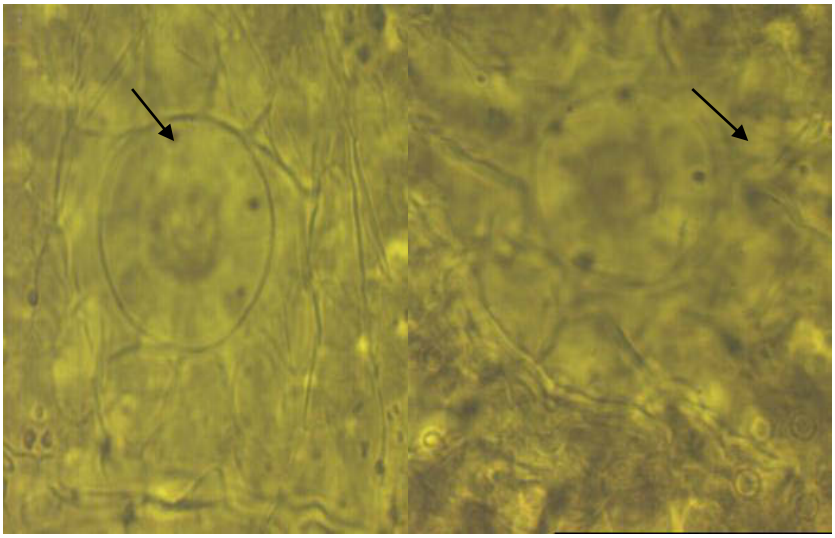
1st node



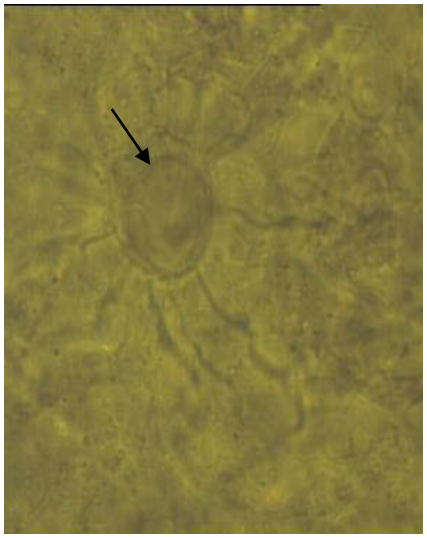
3rd node

5th node

Figure 4: Young, middle and old (1st, 3rd and 5th node)-aged leaf of ***Thymus vulgaris***("Varico 2") treated with 5ppm of GA_3in lower leaf side (arrow indicates the gland size)



1st node 3rd node



5th node

Figure 5: Young, middle and old (1st, 3rd and 5th node) -aged leaf of *Thymusvulgaris*("Deutscher Winter") treated with 1 ppm of GA_3 in lower leaf side (arrow indicates the gland size)

2.8. Determination of the essential oil content

Twelve leaves per treatment were weighed with an electronic high accuracy balance (Sartorius). Then they were transferred to 2ml glass vials and were covered with 20ml of DCM (Dichloride Methane) (Carl Roth, Karlsruhe, Germany). Biphenyl (CAS-Nr92-52-4) (Carl Roth, Karlsruhe, Germany) with a chemical structure $(C_6H_5)_2$, was used as internal standard (ISTD) The covered dried plant material was extracted in an ultrasonic water bath SONOREX RK 156H, Bandelin, Berlin, Germany) for 30 minutes separately for each thyme cultivar.

The components of the essential oil like thymol, carvacrol, linalool, geranial, geraniol, ɣ-terpinene, myrcene, p-cymene, terpinene-4-ol, ß-caryophyllene, geranyl acetate, terpinyl acetate, linalyl acetate, neral, nerol, α-terpineol (%) were determined by gas chromatography. The isolated essential oils were analyzed by GC/FID using a HEWLETT-PACKARD chromatograph, Network 6890N, GC system, (Agilent Technologies, Vienna, Austria) filled with a DB.DB-5 capillary column (10.0m x 0.1mm x film thickness 0.17µm; Agilent Technologies, Vienna, Austria). Injector and detector temperatures were set at 260°C and 300°C. The injection volume was 2µl.The oven temperature was programmed at 60°c for 5 minutes with an increase of 20°C/min until 320°C for 20 minutes. The carrier gas was Helium. For the FID detector, H_2 flow was set to 35 ml/min, air flow to 350ml/min (Split ratio 1:5).

The extracts of 72 samples were analyzed with GC/FID system for analysis. The content of compounds was computed from the GC peak area in relation to the known amount of biphenyl.

3. Results

3.1. Leaf

3.1.1. Leaf weight

Leaf weight was significantly affected by GA_3 in both cultivars “Varico 2” and “Deutscher Winter” as well as in the leaf insertion levels of different age (Figure 6. None of the possible interactions was significant.

Cultivar

The mean difference of “Deutscher Winter” (0.5mg) is higher compared to “Varico 2” (0.4mg).

Leaf age

The first internode showed lowest (0.304 mg) leaf weight; followed by the third (0.538 mg) and fifth which showed highest (0.637 mg) leaf weight, all were significantly different from each other.

GA_3 concentration

The lowest leaf weight (0.438 mg) was observed in thecontrol (0ppm) GA_3 treatment, followed by 1ppm-GA_3with0.521mg and 5ppm-GA_3with0.521mg. All treatments, factors resp.were not significantly different from each other.

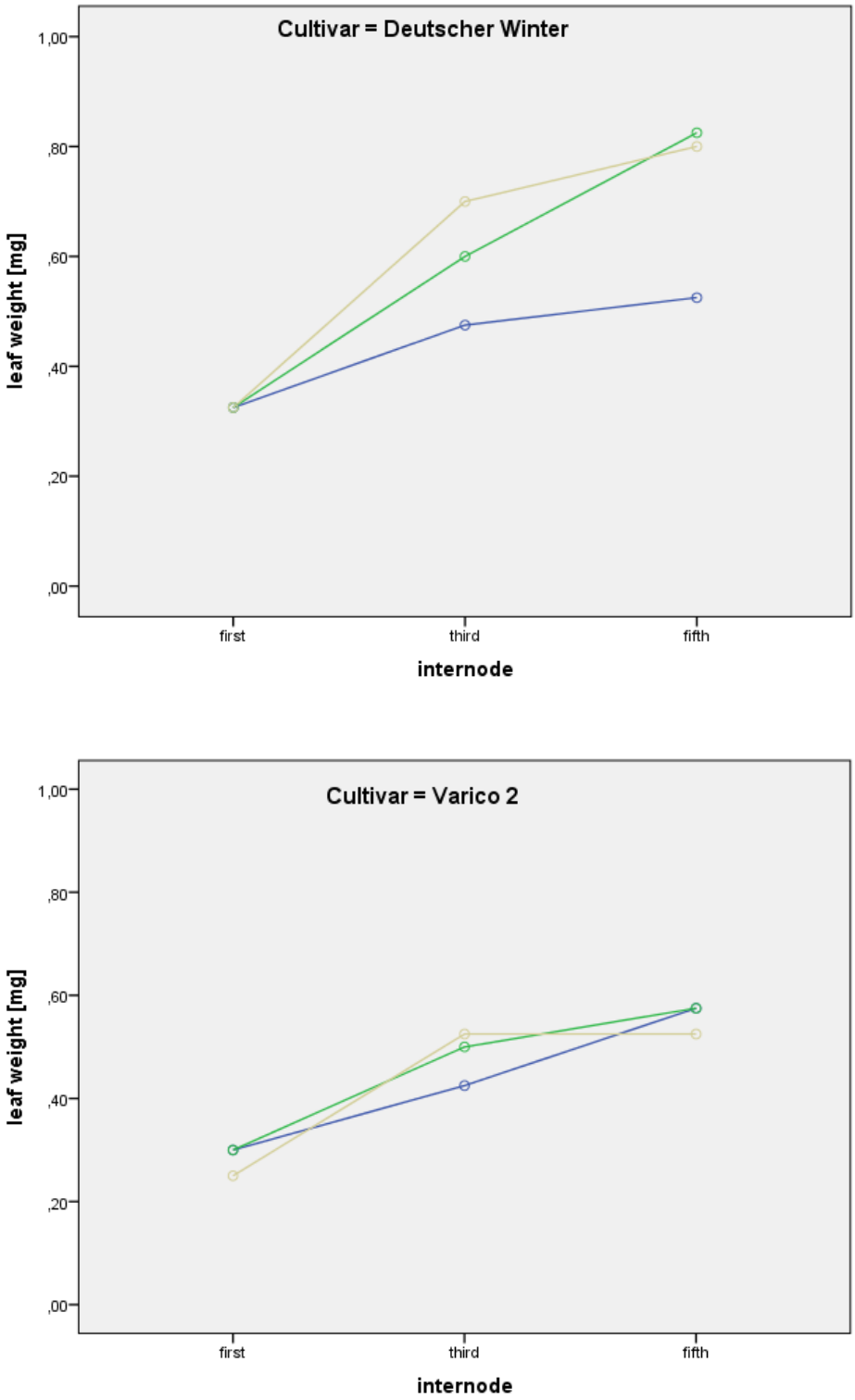


Figure 6: Single leaf weight according to cultivar (upper: “Deutscher Winter”, lower: “Varico 2”), insertion level (internodes 1, 3 and 5) and gibberellin concentration (blue line: 0 ppm; green line: 1 ppm; yellow line: 5 ppm).

3.1.2. Leaf - area

Cultivar, age and gibberellin showed a significant effect on leaf area. The interaction effect between cultivar*age and age*gibberellin also was statistically significant. But, cultivar*gibberellin and cultivar*age*gibberellinwerenotofsignificant effect on leaf area.

Cultivar

The mean value 15.87 mm² (“Deutscher Winter”) is higher compared to 10.15 mm² (“Varico 2”).

Leaf age

The younger leaves were lower (9.98 mm²) which was significantly smaller than the medium and old leaves. Medium (13.69 mm²) and old leaves (15.37 mm²) were not significantly different from each other.

GA_3 concentration

Control (0 ppm GA_3) showed a smaller average leaf area (11.85 mm²) than the treatment with 1ppm (14.76 mm²). Interestingly, the area with treatment 5 ppm-GA_3 resulted in a leaf area (12.43 mm²) between control and 1 ppm-GA_3treatment.

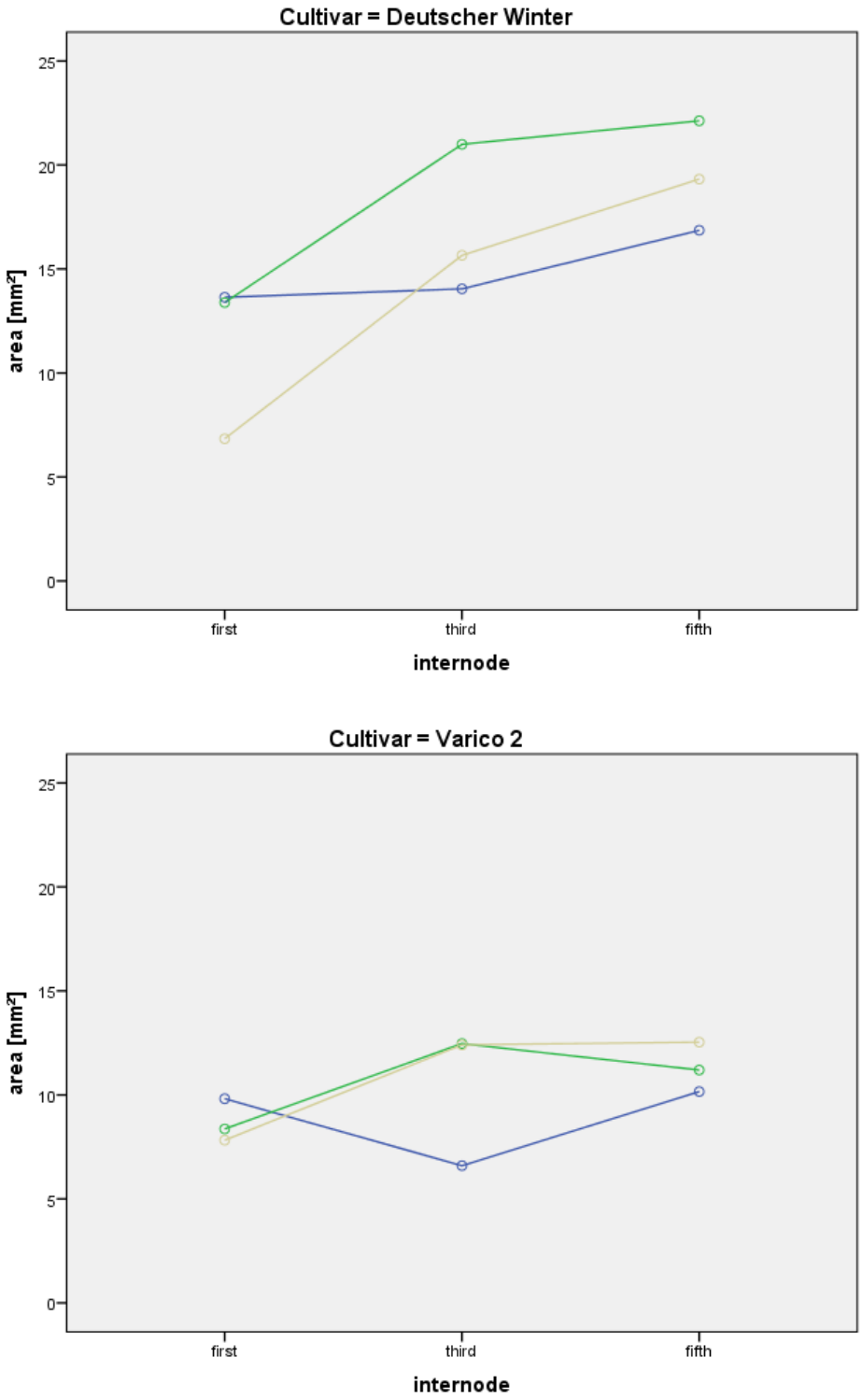


Figure 7: Leaf area according to cultivar (upper: “Deutscher Winter”, lower: “Varico 2”), insertion level (internodes 1, 3 and 5) and gibberellin concentration (blue line: 0 ppm; green line: 1 ppm; yellow line: 5 ppm).

3.1.3. Perimeter

Cultivar, age and GA_3had significant effect on leaf perimeter. The interaction effect between cultivar*gibberellin and age*gibberellin had also a significant effect on leaf perimeter but cultivar *age and cultivar*age*gibberellin had no significant effect.

Cultivar

The mean value of“Deutscher Winter” (20.30 mm) is higher than “Varico2” (16.04 mm).

Leaf age

The young leaves were significantly lower (15.39 mm) than medium (18.92 mm) and old leaves (20.20 mm), which were not significantly different from each other.

GA_3concentration

The highest leaf perimeter (20.74 mm) was observed with the application of 1ppm of gibberellic acid, which was a statistically significant effect. On the other hand, the smallest leaf perimeter (16.43 mm) was recorded on the application of control (0ppm) GA_3 while the application of 5 ppm GA_3 (17.33 mm) not statistically significant from the control.

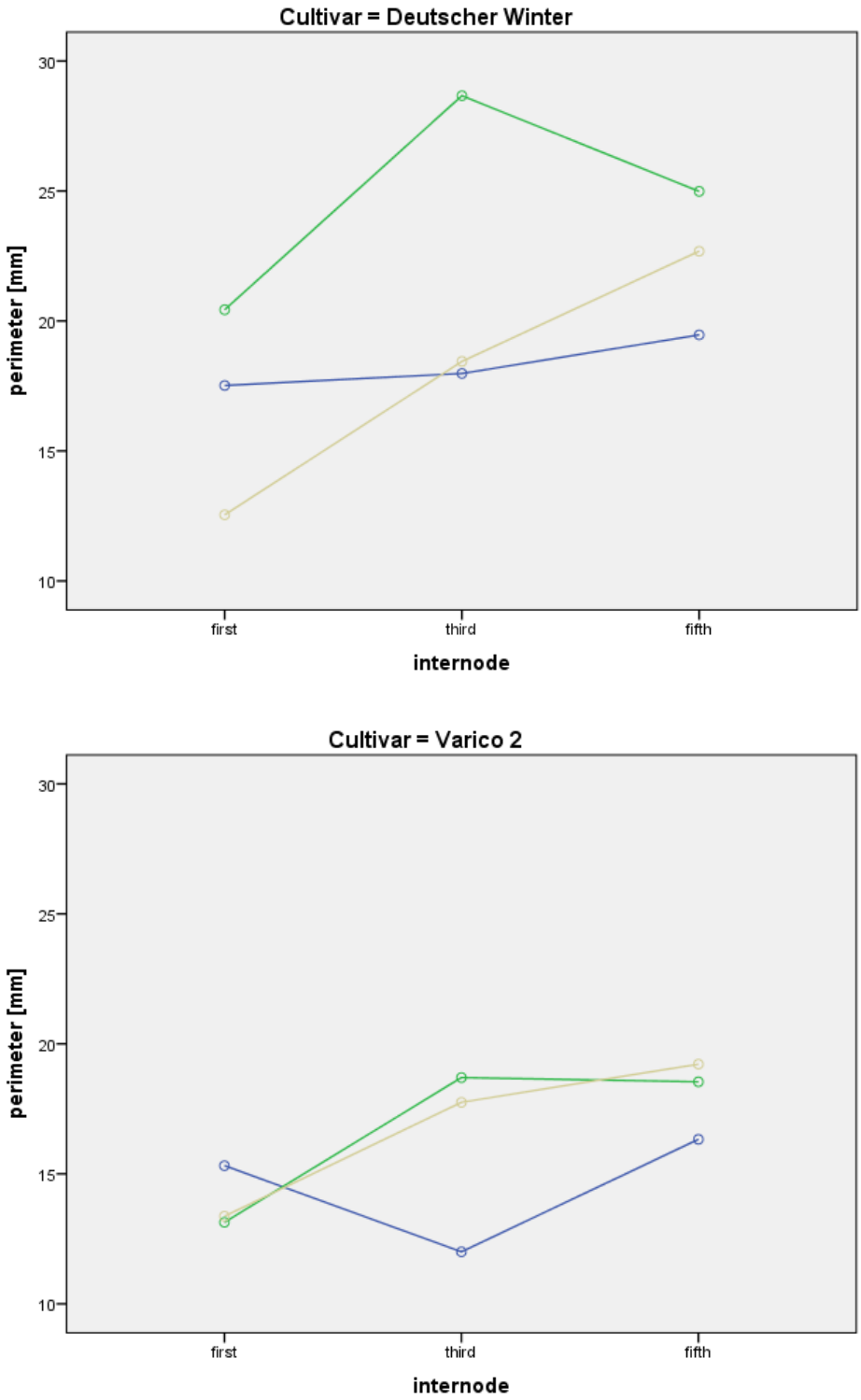


Figure 8: Perimeter according to cultivar (upper: “Deutscher Winter”, lower: “Varico 2”), insertion level (internodes 1, 3 and 5) and gibberellin concentration (blue line: 0 ppm; green line: 1 ppm; yellow line: 5 ppm).

3.1.4. Leaf length

Leaf length varied significantly on cultivar and age. But gibberellins had no significant effect on leaf length. The interaction effect between age*gibberellins had significant effect but cultivar*age, cultivar*gibberellin and cultivar*age*gibberellins were not significant on leaf length.

Cultivar

The mean valueof leaf-length of “Deutscher Winter” (6.93mm) is higher than that of “Varico 2” (6.13mm).

Leaf age

The younger leaves showed lowest leaf length (5.65mm) which was statistically significant compared to medium (6.61mm) and old leaves (7.34mm). The old leaves showed highest leaf length (7.34mm) but not significantly different from others.

GA_3 concentration

The lowest leaf length (6.22mm) was found with the control (0ppm) treatment and the highest leaf length (6.95mm) was observed with the (1ppm) treatment. The leaf length (6.43mm) with treatment with 5ppm GA_3 showed between control and 1ppm. All of them were not statistically significant from each other.

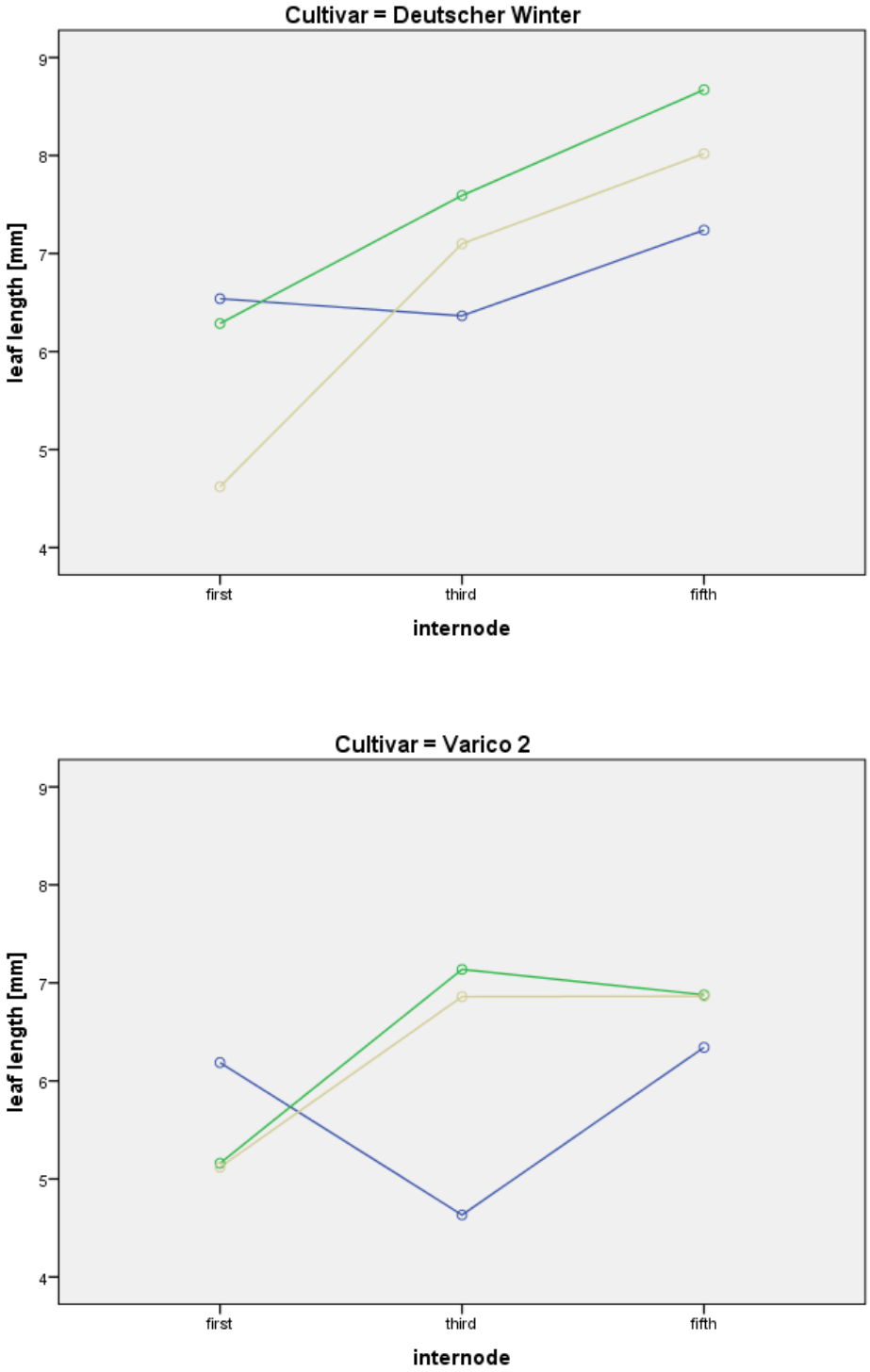


Figure 9: Leaf length according to cultivar (upper: “Deutscher Winter”, lower: “Varico 2”), insertion level (internodes 1, 3 and 5) and gibberellin concentration (blue line: 0 ppm; green line: 1 ppm; yellow line: 5 ppm).

3.1.5. Leaf width

The factors cultivar, age and gibberellins had significant effects on leaf width. The interaction between cultivar*age and cultivar*gibberellins also had significant effect. No significant effect between the interaction of age* gibberellins were determined but a little bit of changes on leaf width. Cultivar*age*gibberellins had no significant effect.

Cultivar

The mean value “DeutscherWinter”(2.84) was higher compared to“Varico 2” (2.05).

Leaf age

The young leaves showed lowest leaf width (2.19mm) which was statistically significant, followed by medium (2.52mm) and old (2.62mm) leaf width. The old leaves showed highest leaf width which was not statistically significant with medium leaves.

GA_3 concentration

The lowest leaf width (2.32mm) was observed with control (0 ppm GA_3) treatment and the highest leaf width (2.63mm) was observed with (1ppm GA_3) treatment. 5 ppm GA_3 treatment showed (2.34mm) leaf width which was not significantly different from others.

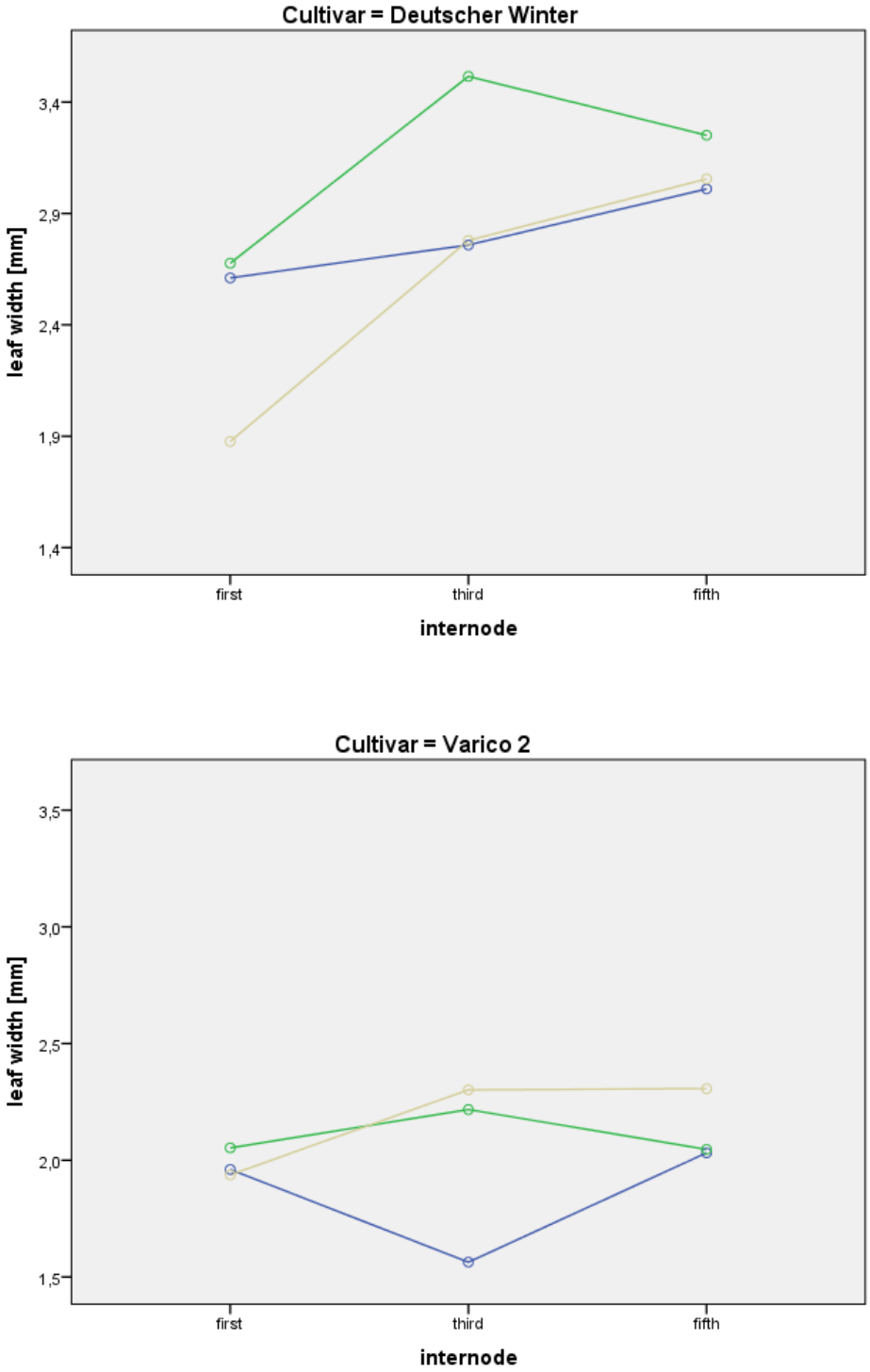


Figure 10: Leaf width according to cultivar (upper: “Deutscher Winter”, lower: “Varico 2”), insertion level (internodes 1, 3 and 5) and gibberellin concentration (blue line: 0 ppm; green line: 1 ppm; yellow line: 5 ppm).

3.2. Glands

3.2.1. Number of glands on the upper leaf side

The factor “cultivar” had significant effect but “age” and “gibberellins” had no significant effect on number of glandsonthe upper leaf side. The interaction between cultivar* gibberellins had significant effect but cultivar*age, age*gibberellins and cultivar*age*gibberellins had no significant effect on number of glandsonthe upper leaf side.

Cultivar

The mean value of “Deutscher Winter” (132) is higher than that of “Varico 2” (87).

Leaf age

The young leaves showed lowest number of glandson the upper leaf side(102) and old leaves showed 105 glands. Medium leaves highest (122), which were not significant from each other.

GA_3 concentration

The lowest (103) number of glandson the upper leaf sidewere observed with higher concentration (5ppm GA_3) compared to (112) with 1ppm GA_3. Interestingly, the highest (114) number of glandson the upper leaf sidewas observed with control (0 ppm GA_3) which was not significant from each other.

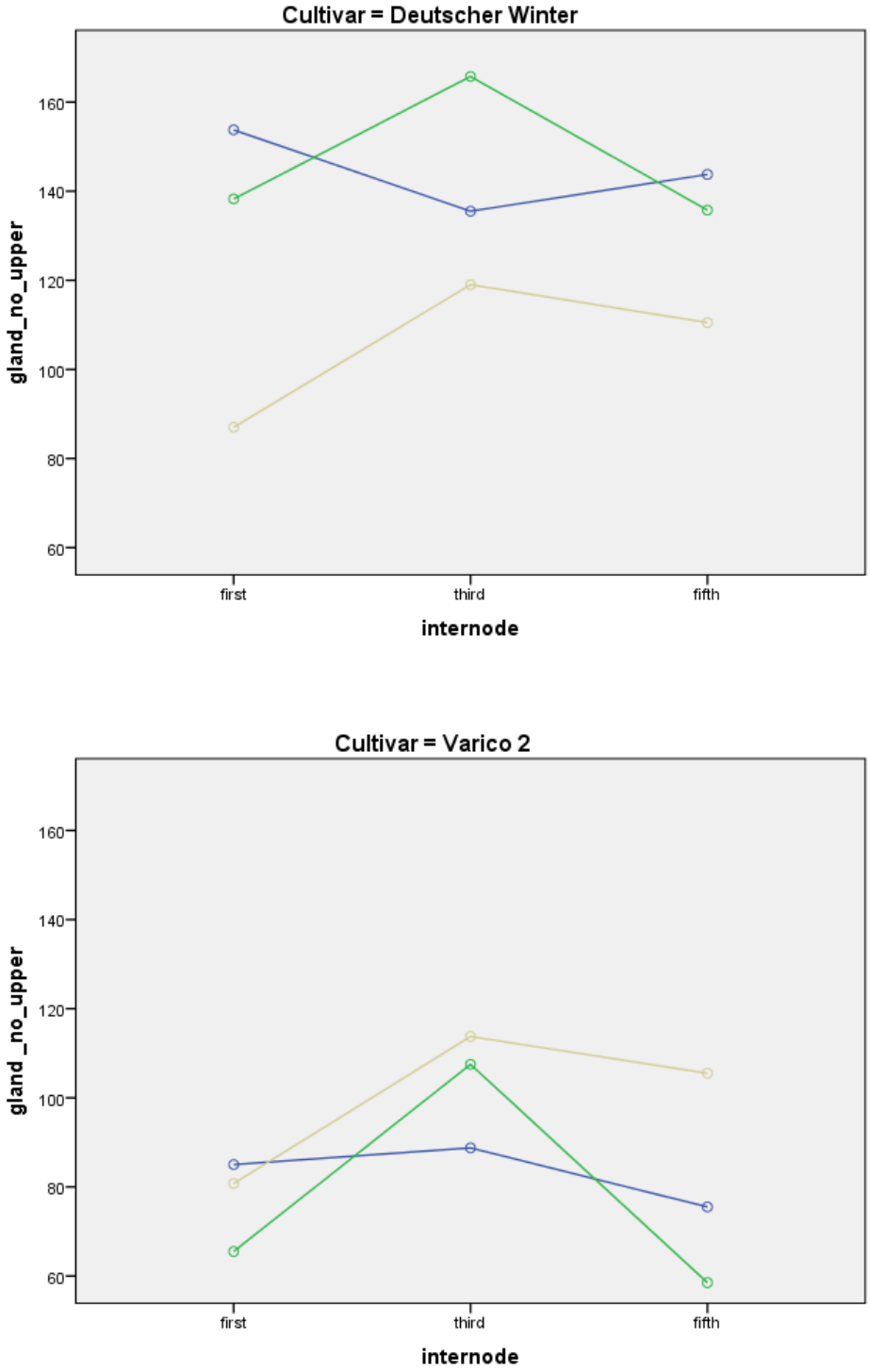


Figure 11: Number of glands on the upper leaf side according to cultivar (upper: “Deutscher Winter”, lower: “Varico 2”), insertion level (internodes 1, 3 and 5) and gibberellin concentration (blue line: 0 ppm; green line: 1 ppm; yellow line: 5 ppm).

3.2.2. Number of glands on the lower leaf side

The cultivar had significant effect but age; gibberellins had no significant effect on the number of glandsonthe lower leaf side. There was a significant effect between cultivar*gibberellin but cultivar*age, cultivar*age*gibberellins had no significant effect on the number of glands onthe lower leaf side.

Cultivar

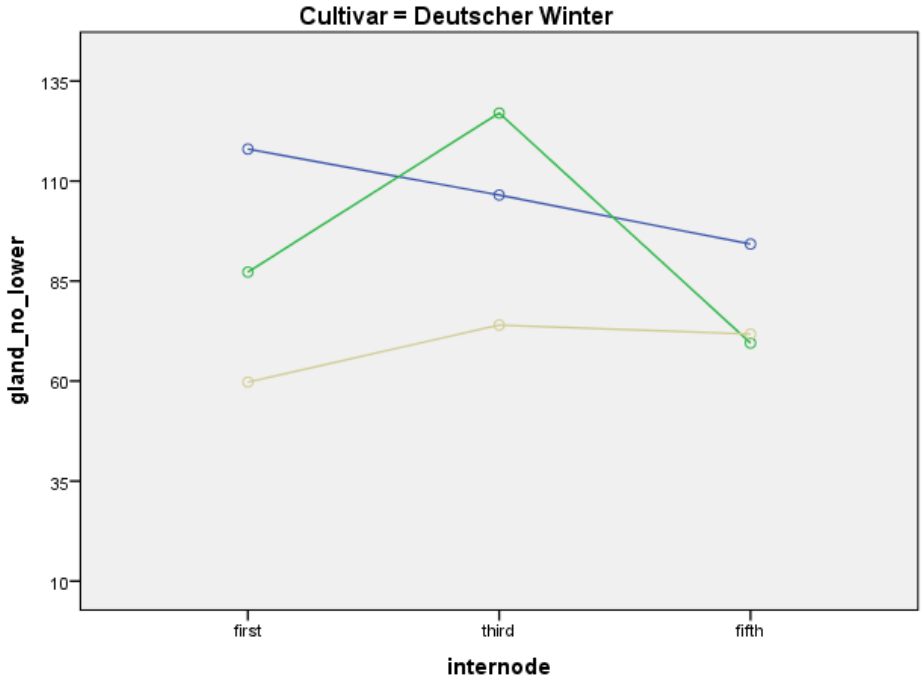
The mean value of “DeutscherWinter” (90 glands) is higher compared to “Varico 2” (42 glands).

Leaf age

On the lower sidethe old leaves showed the minimum number of glands (59) and medium leaves showed maximum number of glands (75). The young leaves with an average of 64 glands were not significantly different from the old or the medium leaves.

GA_3 concentration

The minimum number of glands (59)onthe lower leaf side were found with the medium concentration (1ppm GA_3) treatment compared to (63) with 5ppm GA_3 and interestingly, the maximum number ofglands(76) onthe lower leaf side was observed in thecontrol (0ppm GA_3) treatment. Results concerning the concentrations were not statistically significantlydifferent from each other.



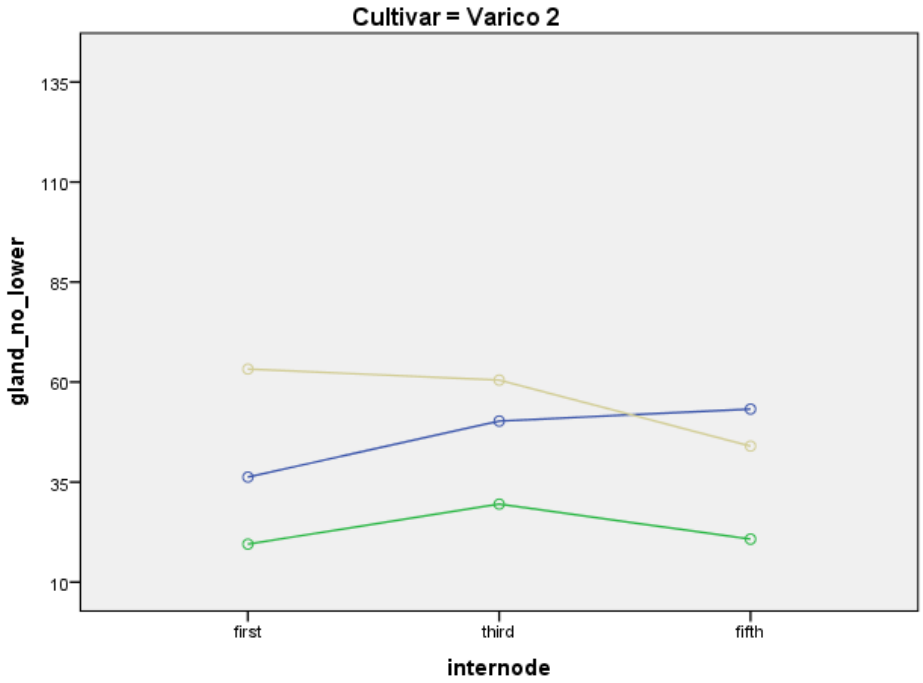


Figure 12: Number of glands on the lower leaf side according to cultivar (upper:“Deutscher Winter”, lower:“Varico 2”), insertion level (internodes 1, 3 and 5) and gibberellin concentration (blue line: 0 ppm; green line: 1 ppm; yellow line: 5 ppm).

3.2.3. Number of glands per mm² on the upper leaf side

Cultivar, age and gibberellin had no significant effect on number of glandsper mm^2 on the upper leaf side. The interaction effect between cultivar*age, cultivar*gibberellins, age*gibberellins and cultivar*age*gibberellin also had no significant effect on number of glandsper mm^2 on the upper leaf side.

Cultivar

The mean value“Deutscher Winter” (10) is lower compared to“Varico 2” (11).

Leaf age

The old leaves were observed minimum (7) number of glands per mm² and medium leaves showed highest (11) number of glands per mm² on the upper leaf side which were not statistically significant from each other. The young leaves showed number of glands per mm² (11).

GA_3concentration

The minimum (8) number of glandsper mm²on the upper leaf side were recorded with medium concentration (1ppm GA_3), followed by (9) showed with higher concentration (5ppm GA_3) and interestingly, the maximum (12) number of glands per mm² on the upper leaf side showed control (0ppm GA_3) treatment which were not significant from each other.

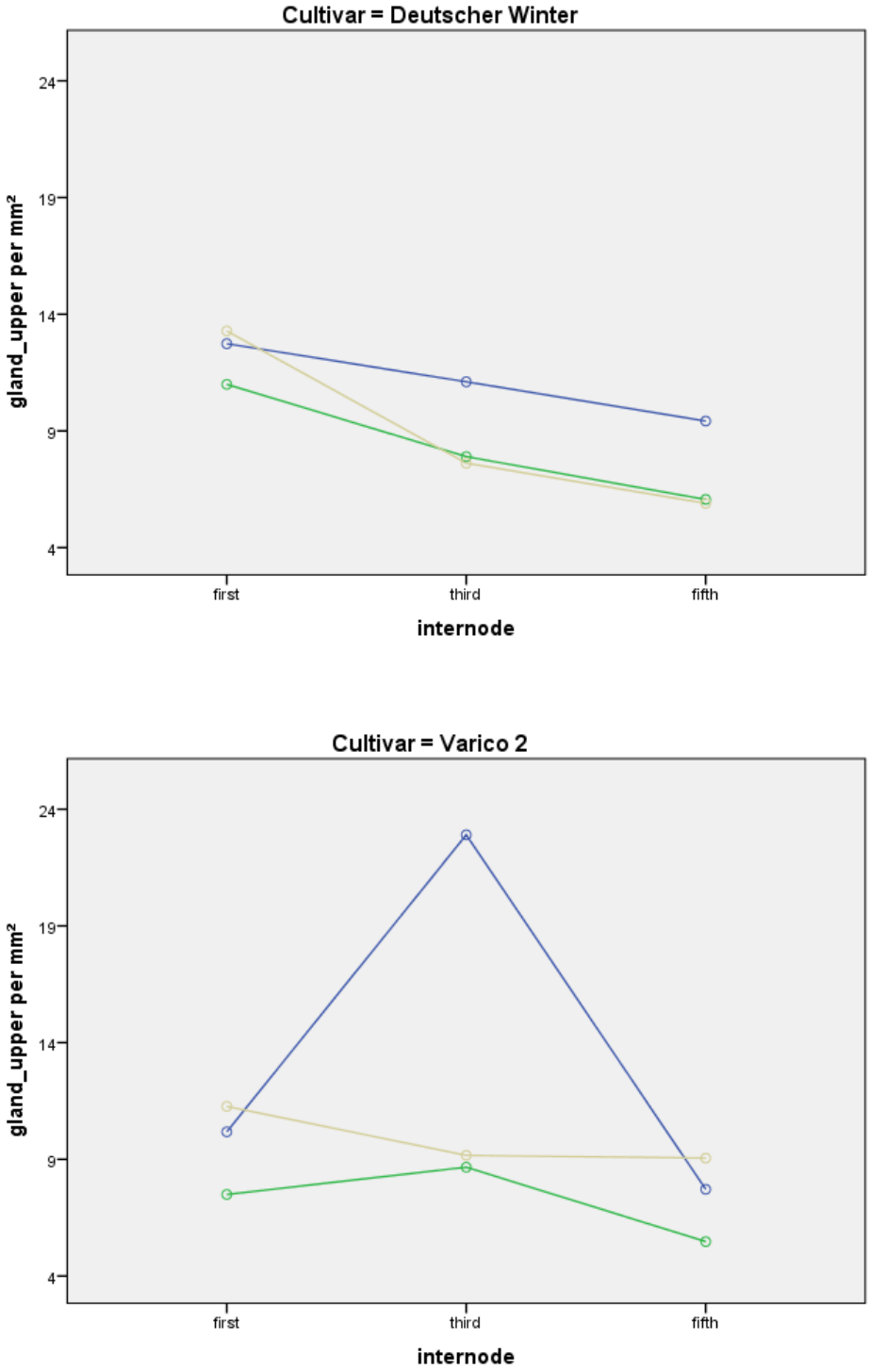


Figure 13: Number of glands on the upper leaf side per mm² according to cultivar (upper: “Deutscher Winter”, lower: “Varico 2”), insertion level (internodes 1, 3 and 5) and gibberellin concentration (blue line: 0 ppm; green line: 1 ppm; yellow line: 5 ppm).

3.2.4. Number of glands per mm² on the lower leaf side

Age and gibberellins both had a significant effect but cultivar had no significant effect on number of glands on the lower leaf side per mm². The interaction between cultivar*age, cultivar*gibberellins, age*gibberellins and cultivar*age*gibberellins were not significant effect on number of glands on the lower leaf side per mm².

Cultivar

The mean value “Deutscher Winter” (6) is higher compared to “Varico 2” (5).

Leaf age

The old leaves showed minimum (4) number of glands on the lower leaf side per mm² and young leaves showed highest (7) which were not significant from each other. The medium leaves showed (7) which were between young and old.

GA_3 concentration

The minimum (4) number of glands on the lower leaf side per mm² was observed with the 1ppm-GA_3 treatment followed by (6) which was the5ppm-GA_3 treatment. Interestingly, the maximum (8) number of glands on the lower leaf side per mm² was exhibited by the control (0 ppm GA_3).

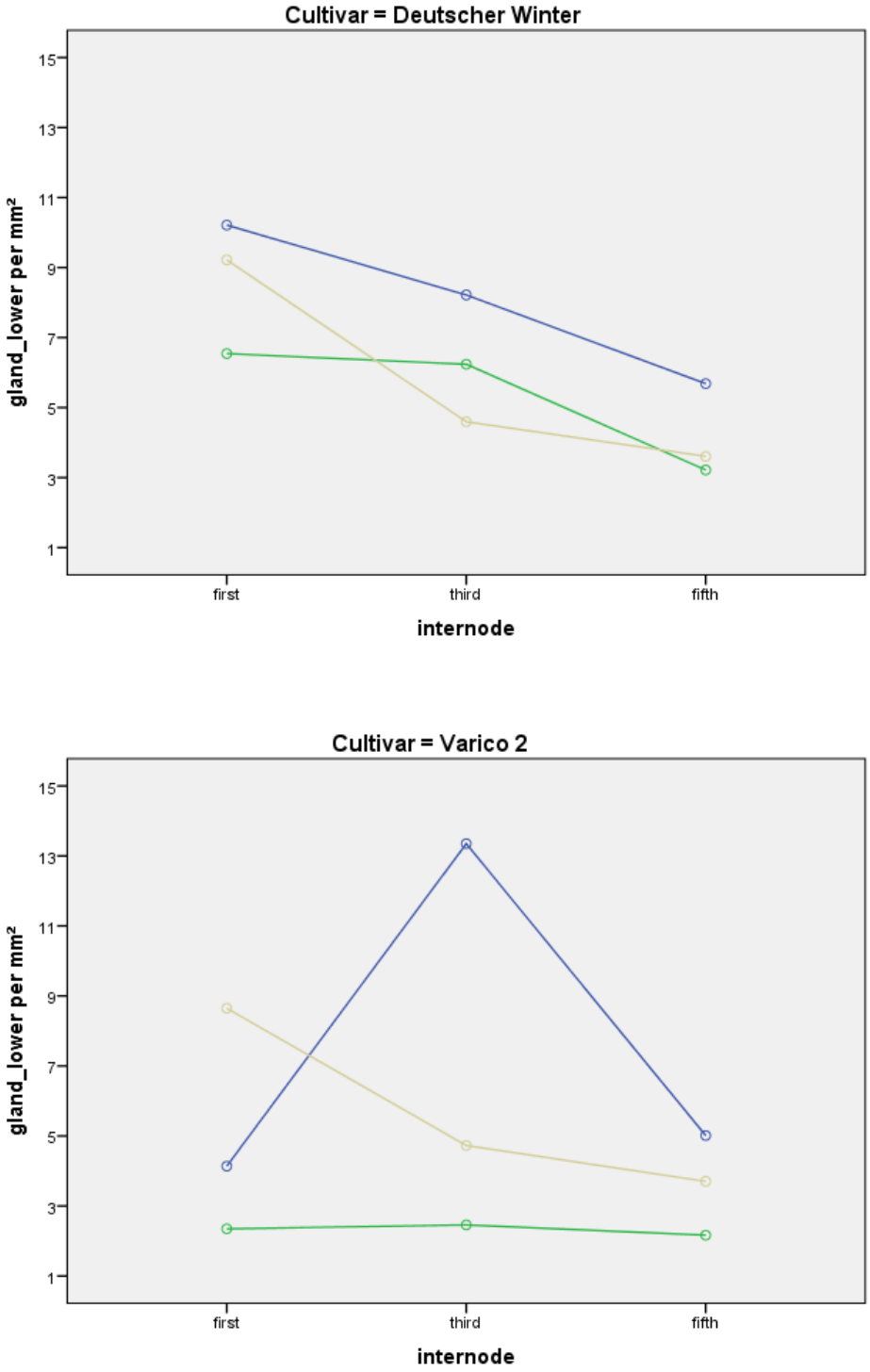


Figure 14: Number of glands on the lower leaf side per mm² according to cultivar (upper: “Deutscher Winter”, lower: “Varico 2”), insertion level (internodes 1, 3 and 5) and gibberellin concentration (blue line: 0 ppm; green line: 1 ppm; yellow line: 5 ppm).

3.2.5. Mean gland size

Factors cultivar, age and gibberellins had no significant effect on mean gland size. The interactions between cultivar*age, cultivar*gibberellins, age*gibberellins and cultivar*age*gibberellins were not significantly effective on mean gland size.

Cultivar

The mean value “Deutscher Winter” (3180) is lower compared to the one of “Varico 2” (3246).

Leaf age

The old leaves showed smallest gland size (3142 µm²) and medium leaves showed largest gland size (3330µm²). The young leaves showed (3166µm²) in between old and medium.

GA_3concentration

The smallest gland size (3178µm²) was observed with (5 ppm GA_3)-treatment compared to 3184µm²of thecontrol (0 ppm GA_3) treatment and the largest gland size (3275µm²) was found with (1 ppm GA_3)-treatment.

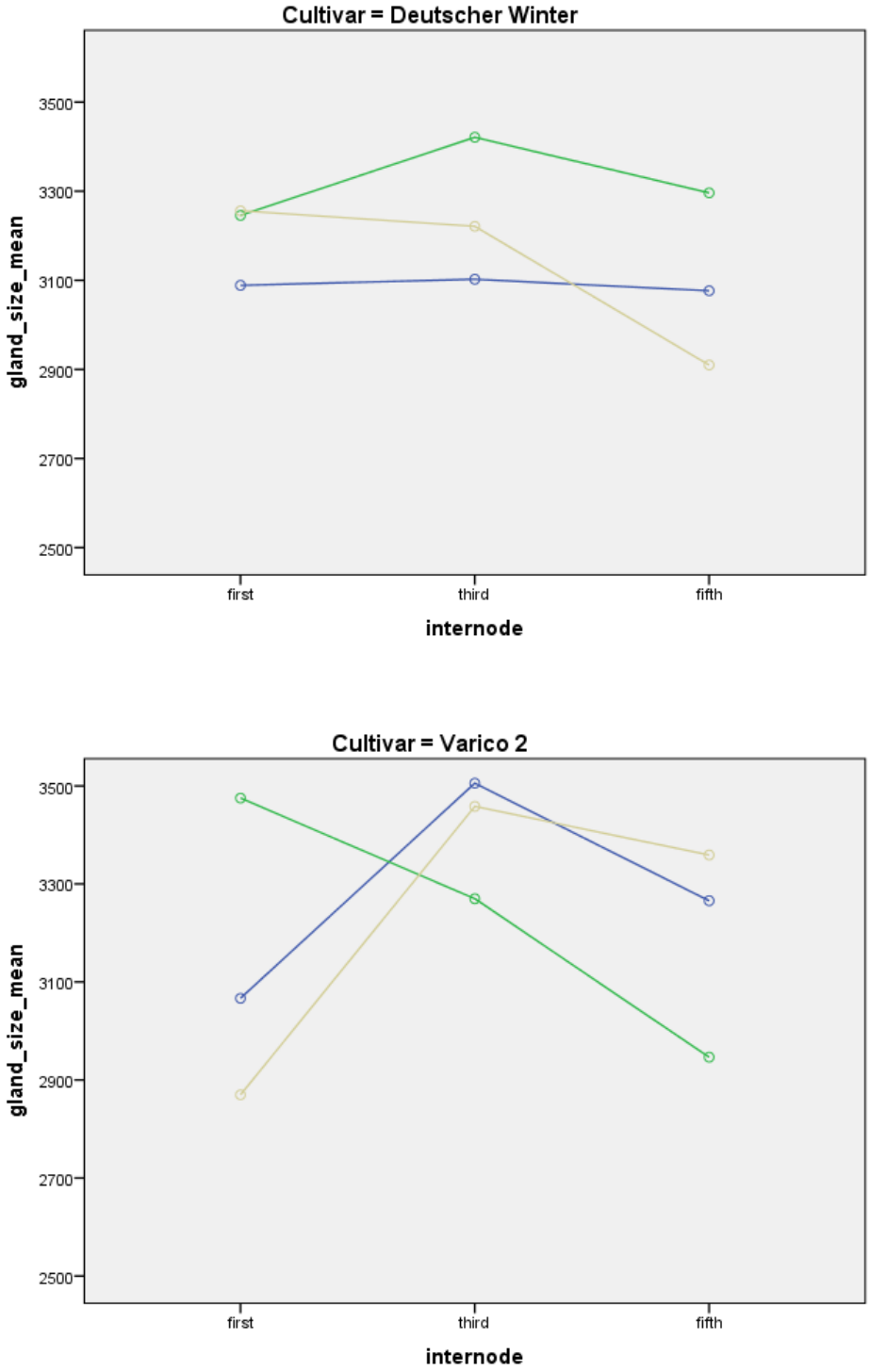


Figure 15: Mean gland size according to cultivar (upper: “Deutscher Winter”, lower: “Varico 2”), insertion level (internodes 1, 3 and 5) and gibberellin concentration (blue line: 0 ppm; green line: 1 ppm; yellow line: 5 ppm).

3.3. Essential oil content (%)

Essential oil contents varied significantly depending on cultivar, leaf age and gibberellin-treatments (Figure 11). There was also a significant effect in the interaction between cultivar*leaf age. Cultivar*gibberellin, Leaf age*gibberellin and cultivar*leaf age*gibberellin did not show a significant effect on essential oil content.

Cultivar

The mean value of essential oil content of“Deutscher Winter”(1.68%) is lower compared to “Varico 2” (3.63%).

Leaf age

The young leaves showed lowest amount of essential oil (2.34%), old leaves (2.53%) and medium leaves showed highest amount (3.10%) of essential oil which was not significant from others.

GA_3 concentration

The lowest amount (2.16%) of essential oil was observed with (5 ppm GA_3) treatment and interestingly, the highest amount (3.07%) of essential oil was observed with control (0 ppm GA_3) treatment. Essential oil content of (2.75%) with the treatment 1ppm-GA_3 was observed between 5ppm and control.

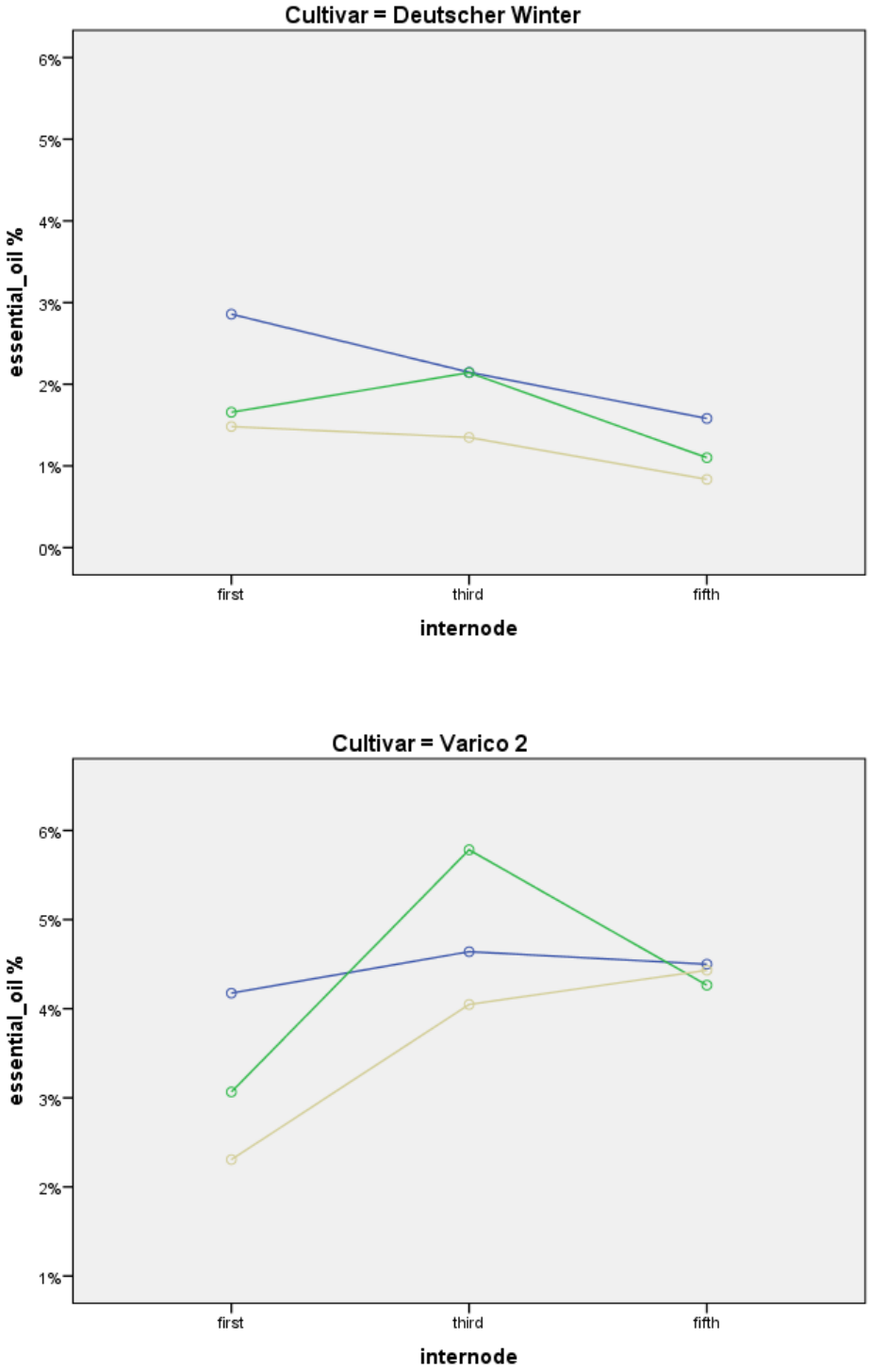


Figure16: Essential oil (%) according to cultivar (upper: “Deutscher Winter”, lower: “Varico 2”), insertion level (internodes 1, 3 and 5) and gibberellin concentration (blue line: 0 ppm; green line: 1 ppm; yellow line: 5 ppm).

3.4. Correlation between variables

It is of importance to know which of the measured variables (some of them yield components) correlates well to the essential oil content. Therefore, we show here only the dependency of the essential oil content to all other variables (as possible yield components of the essential oil content) (Table 1).

Table 1: Correlation coefficients (r) between essential oil content and the other variables by cultivar.

Variable	**Deutscher Winter**		**Varico 2**	
	R	Sig.	r	Sig.
Leaf weight	-0.313	n.s.	0.289	n.s
Area	0.023	n.s	0.088	n.s
Perimeter	0.191	n.s		n.s
Leaf length	0.088	n.s	0.072	n.s
Leaf width	-0.042	n.s	0.057	n.s
No. of glands (upper leaf side)	0.368	*	0.478	*
No. of glands (lower leaf side)	0.454	*	0.230	n.s
No. of glands (upper leaf side per mm²)	0.221	n.s	0.262	n.s
No. of glands (upper leaf side per mm²)	0.311	n.s	0.162	n.s
Mean Gland size	-0.052	n.s	0.354	*

Correlation-calculation was done in order to determine the relation between of the essential oil with all other variables. From the correlation table (Table 1) it can beobserved that in “Deutscher Winter” there were no significant correlations with essential oil except “number of glands” on the upper leaf side and “number of glands” on the lower leaf side. Number of glands on the upper side (r = 368*) and

number of glands on the lower side (r = 0.454*) showed medium positive correlations with the essential oil content that were weakly significant.

In “Varico 2” there were no significant correlations observed except number of glands on the upper leaf side and mean gland size. Number of glands on the upper leaf side (r = 0.478*) and mean gland size (r = 0.354*) showed medium positive correlations which were significant.

4.Results

GA_3 in high concentrations (30 ppm) resulted in much smaller and thinner leaves and lower gland density in the closely related oregano (*Origanum* x *intercedens*)

(Bosabailidis and Exarchou, 1995). Therefore, we have chosen with 8 ppm GA_3 a maximum concentration where no first visual effects on leaf size could be observed.

4.1. Leaf morphology

Thymus vulgaris has very small but numerous leaves. In average, dry weight of a typical leaf is around 0.5 mg with a mean area around 10 mm² to16 mm². The effect of GA_3 on leaf morphology in general was not prominent in both cases (“Deutscher Winter” and “Varico 2”) and weaker than expected.The 1st internode showed lowest leaf weight with the higher concentration of GA_3 in both cultivars (”Deutscher Winter” and “Varico 2”). On the other side, 5th internode of both cultivars decreased with increasing concentration of GA_3.

Similar results were reported by Antypas (2015). Respecting the morphological measurements such as the area, length, width and weight of the dry leaves, the changes were not significant. As GA_3 is a plant growth regulator, so a bigger size of leaves was expected which is not the case in our results. Analogous results were found by Schmiderer et al. (2010) in the morphology of the leaves of common sage (*Salvia officinalis*) after foliar application of 0.8ppm and 8 ppm of GA_3. In that case the application of daminozide of 500ppm and 5000ppm respectively, a plant growth retardant and an inhibitor of gibberellin synthesis, resulted to greater leaf length, leaf width and leaf area. In this research we observed, that the higher concentration of GA_3that were applied, resulted in lower leaf-area, leaf-perimeter, leaf-length and -width. Similar results were reported by Bosabalidis and Exarchou(1995) the treatment of *Origanumxintercedens* with GA_3 caused a significant decrease in the mean surface area of the leaf blade. In contrast, Anasari et al. (1988) found that GA_3 increased the size of the leaf blade of *Cymbopogonjawarancusa*, another plant producing essential oil.

4.2. Glands

The plants produced around 132 and 87 glands on the upper leaf side (“Deutscher Winter” and “Varico 2”, respectively) and 90 and 42 glands on the lower leaf side (again for“Deutscher Winter” and “Varico 2”, respectively. In density that is 9 and 10

glands per mm² (upper leaf side, “Deutscher Winter” and “Varico 2”, respectively) and 6 and 5 glands per mm² on the lower side (“Deutscher Winter” and “Varico 2,” respectively). The two thyme cultivars demonstrated the different results regarding the number of oil glands in the leaves. In this research, when the higher concentrations of GA_3 were applied the lowest number of glands were reported for the total number of glands per leaf.Analogous results were reported by Antypas (2015). An insignificant decrease with the application of1 ppm of GA_3 in “Deutscher Winter” was observed but in general, there was no influence to the density of oil glands by GA_3. The most remarkablefindingwas the different distribution of the glands in the leaves.The application of GA_3 influenced not only the density of the glandular trichomes on the surfaces of the *Origanum x intercedens*leaves, but also their size. GA_3 resulted in increased head diameter for the trichomes on the upper leaf surface but in decreased head diameter on the lower surface (Bosabalidis and Exarchou, 1995).

It is of high importance to mention that in this research, the abaxial side of the leaf was observed where mainly the type II capitate glandular hairs are located. These glands were randomly distributed all over the leaves amongst the nodes. The secretory materials are released soon after their production when the cuticle ruptures or through the cuticle via droplets. It has been observed that type II capitate glandular hairs have a thicker cuticle cover than the epidermal one (Bisio et al. 1999). Typically, there are plants with high and low levels of secreting materials and the latter are those plants which form mainly exudates such as mono-terpenes in principal like Thymus. These lower terpenes are highly volatile and they characterized by the extent of their catabolism and reutilization, factors which may play a major role in the amount of product amassed. When the cuticle ruptures, volatilized secretion material might escape. Thus, it seems to be no correlation between the exudate accumulation capacity and the diameter and the size of the glands (Wagner, 1991)

4.3. Essential oil content

Essential oil contents of *Thymus vulgaris*cultivars were 1.68% and 3.63% (“Deutscher Winter” and “Varico 2” respectively).A remarkable increase was

observed in the essential oil content of middle-aged and old–aged leaves of “Deutscher Winter” and “Varico 2” of *Thymus vulgaris* L. species.Similar results were reported by Antypas(2015).He also reported that the higher the concentration of GA_3, the higher the essential oil content. In the case of “Deutscher Winter”, young and middle-aged leaves had the same essential oil content after the treatments while the old leaves showed to contain more than the other two leaf ages. Butinterestingly in this research, when the higher concentration (5 ppm) of GA_3 was applied, the lowest amount of essential oil was observed. The similar results were reported by Reda et al. (2005).The young leaves contain the lower amount of essential oil. It has been already reported by Sangwan et al. (2001) that foliar application of active GA_3 is a stimulus of increase of the essential oil compounds in many species for some economically important essential oil crops such as*Pelargonium sp*,*Ocimum sanctum*, *Ocimumbasilicum*, *Cymbopogonjwarancusa*and *Artemisia annua*. It is found that there is a co-regulation between mRNA and many enzymes with consistent protein and more specifically an up-regulation between enzymes related to production of terpenoids and mRNA levels. This could be a reason for the increase of essential oil content (Xie et al., 2008). There might be a direct influence of the internal levels of active GA_3 to the promoter except from enzymes in the metabolic pathways, and deviations in the correlation between mRNA and metabolite levels might be due to post-transcriptional, and/or post-translational modifications and/or further metabolism of the monoterpenes (Schmiderer et al., 2010).

Generally, “Deutscher Winter” and “Varico 2” both thyme cultivars are thymol-chemotype cultivars, which means that they have high essential oil yield performance, especially thymol. In this research the main componentThymolwith 70% was observed. It has been found by Bagdat et al. (2015) and Baher et al. (2002) that the amount of carvacrol and thymol was increased in contrast with γ-terpene that was decreased. According to Carlen et al. (2009) who compared the new *Thymus vulgaris* L. hybrid cultivar “Varico 3” to five established cultivars from

Germany, France and Switzerland including “Deutscher Winter”, above all was “Varico 3” respecting the essential oil content and the lowest was “Deutscher Winter”. It has been demonstrated by Antypas (2015) that an old cultivar *Thymus*

vulgaris L. (Varico 1) with low essential oil yield performance and a cultivar *Thymus vulgaris* L. (DeutscherWinter) with high oil yield were picked.

5.Conclusion

In this study, we were investigating the effect of GA_3on the production of essential oil in thyme (*Thymus vulgaris L.,* Lamiaceae). Parameters of leaf morphology wereanalyzed by image analysis, epidermal essential oil glands counted under the microscope and the essential oil content determined by gas chromatography.

From this research, the most interesting result has been reported that applied with the higher concentration (5 ppm) of GA_3 the lowest amount of essential oil was observed for both cultivars ("Deutscher Winter" and "Varico 2"). It has been demonstrated by previous research that GA_3 is one of the most important plant growth regulators and it can stimulatean increase of mono-terpenes in essential oil crops and several but not so effective in inducingmorphological changes of the plant. In this study, the two thyme cultivars "Deutscher Winter" and "Varico 2" of thyme showed that there were no significant influences of GA_3 concentrations on the essential oil content. Actually a decrease had to be noticed.

The production of the essential oils in medicinal and aromatic plants and specifically in Lamiaceae family is regulated by diverse physiological and biochemical processes genetics and metabolic pathways. The EOs are used as a flavor additive, to improve the quality of a cosmetic product as well as antimicrobial and antioxidativeside-effects in products and as a fundamental olfactoricprinciple for consumers. Therefore, essential oil production should be increased for its diverse use in pharmacopoeias of Europe, Germany and United Kingdom and used as natural preservatives in the food industry. From the overall result it was found "Varico 2" showed high amount (3.63%) of essential oil content compared to "Deutscher Winter" (1.68%). In general, hybrid cultivars showed a higher homogeneity than the population cultivar "Deutscher Winter". In conclusion, of the research work indicate that hybrid cultivar "Varico 2" can be recommended for the producers as thymol-chemotype thyme.

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List of Abbreviations

EO's – Essential oils

GA_3 – Gibberellic acid 3

CV- Cultivar

NAA – (1-naphthaleneacetic acid)

IAA – Indole-3- acetic acid

ANOVA – Analysis of Variance

DCM – Dichloromethane

ISTD – Internal standard

GC/FID – gaschromatography