Selection of lupin genotypes with resistance against aphids and its dependence on quinolizidine alkaloid content and composition

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I shall be telling this with a sigh
Somewhere ages and ages hence:
Two roads diverged in a wood, And I-
I took the one less travelled by,
And that has made all the difference.

The Road Not Taken – Robert Frost

Dedicated to all those who made the less travelled road

a worthwhile decision.

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II. List of abbreviations

BBCH	BBCH-Skala (**B**iologische **B**undesanstalt für Land- und Forstwirtschaft, Bundessortenamt und **Ch**emische Industrie)
CuAO	Copper amine oxidase,
d	Days
ECT/EFT-LCT/LFT	ρ-coumaroyl-CoA/ feruloyl-CoA: (+)-epilupinine/(–)-lupinine O-coumaroyl/ feruloyltransferase,
e.g.	Exempli gratia / for example
etc.	Et cetera
EPG	Electrical penetration graph
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
h	Hour(s)
HCL	Hydrochloric acid
HMT/HLT	tigloyl-CoA:(–)-13α-hydroxymultiflorine/(+)-13α-hydroxylupanine O-tigloyltransferase)
i.e.	id est / in other words
L	Liter
L/ODC	Lysine decarboxylase,
mg	Milligram
mm	Millimetres
µg	Microgram
n	Number
NaOH	Sodium hydroxide
NAP	National Action Plan(s)
p	Significance level
QA / QAs	Quinolizidine alkaloid (s)
spp.	Species
°C	Degree Celsius
%	Percent

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Abstract

The cultivation of lupins (*Lupinus* spp.) is of growing interest with respect to its usability in food and feed production, in particular due to its high protein content. Moreover, owing to numerous favourable agronomic characteristics, such as its capability for water acquisition from deeper soil layers, its ability to bind elementary nitrogen and make it biologically available by mutualistic symbiosis with Bradyrhizobiaceae, and its ability for mobilization of additional nutrients, lupins have the potential to widen crop rotations.

The use of lupins, in particular the narrow-leafed lupin (*Lupinus angustifolius* L.), for human consumption and animal feeding is substantially based on breeding of so-called sweet lupins, containing a low amount of quinolizidine alkaloids. Quinolizidine alkaloids (QAs) are toxic secondary metabolites, with the capability to cause severe health issues in humans and animals. Coincidentally, they provide a natural defence against herbivores such as aphids, and by reducing its content in lupins an increased susceptibility for aphid infestation was observed. Hence, breeding of aphid resistant cultivars, along with a low QA content is needed to prevent yield losses and reduce the application of insecticides for aphid control.

For an appropriate breeding success, evaluation of a diverse collection of narrow-leafed lupin genotypes with regard to acceptance as host for aphid species and concerning their QA content and composition is a prerequisite. Therefore, the multiplication of *Macrosiphum albifrons, Aphis fabae, Aphis craccivora, Acyrthosiphon pisum* and *Myzus persicae* as probably most significant aphid pests was investigated on 46 narrow-leafed lupin genotypes under controlled conditions, and the QA composition and total content were analysed. Results indicated that the well-adapted lupin aphid (*M. albifrons*) is able to develop on all genotypes investigated, unaffected by a high or low QA content, respectively. For *A. fabae, A. pisum, M. persicae* and *A. craccivora* a negative correlation between aphid multiplication and total QA content was observed. However, some genotypes were identified, containing a low total QA content but allowing no or only a very limited aphid multiplication. It was shown that not only the total content but the composition of QAs is influencing the aphid multiplication rate, with 13-tigloyoxilupanine showing the most significant effects.

Furthermore, the evaluation of the influence of the lupin genotype on the feeding behaviour of several aphid species as an indicator for the suitability as a host was

conducted to investigate the underlying impact of the QA content. In this regard, the probing and feeding behaviour of the above-mentioned aphid species on four genotypes containing varying amounts and compositions of QAs was observed with the electrical penetration graph (EPG) technique. Results approved the observations of the multiplication trials by showing that *A. fabae, A. craccivora, A. pisum,* and *M. persicae* performed decreased probing in the presence of a high QA content, whereas *M. albifrons* was not negatively influenced. The most significant differences were found in phloem-related parameters. On host plants with a high content or unfavourable composition of QAs a reduced occurrence as well as a decreased duration of phloem related probing phases was observed, except for *M. albifrons*. The genotype (cv. 'Kalya') identified in the multiplication trials as having a low total QA content but reduced aphid susceptibility also showed an influence on the probing and feeding behaviour.

Genotypes identified in the evaluations as having a low total QA content in coincidence with reduced aphid development can be used as the starting point for breeding of sweet narrow-leafed lupin varieties with aphid resistance. The content of 13-tigloyloxilupanine was identified as the criterion which can be used for indirect selection in narrow leafed lupin breeding programs.

Zusammenfassung

Hinsichtlich ihrer Verwendbarkeit in der Lebensmittel- und Futtermittelproduktion ist der Anbau von Lupinen (*Lupinus* spp.) von wachsendem Interesse, insbesondere aufgrund ihres hohen Proteingehalts. Aufgrund zahlreicher günstiger agronomischer Eigenschaften, wie beispielsweise der Fähigkeit zur Gewinnung von Wasser aus tiefer liegenden Bodenbereichen, der Fähigkeit durch mutualistische Symbiose mit Bradyrhizobiaceae elementaren Stickstoff zu binden und biologisch verfügbar zu machen, und der Fähigkeit zur Mobilisierung weiterer Nährstoffe, ist der Einsatz von Lupinen zur Erweiterung der Fruchtfolge vielversprechend.

Die Verwendung von Lupinen, insbesondere der schmalblättrigen Lupine (*Lupinus angustifolius* L.), für den menschlichen Verzehr und in der Tierernährung beruht im Wesentlichen auf der Züchtung von sogenannten Süßlupinen, die nur eine geringe Menge von Quinolizidinalkaloiden enthalten. Quinolizidinalkaloide (QAs) sind toxische Sekundärmetabolite, die bei Menschen und Tieren schwere gesundheitliche Probleme verursachen können. Zugleich bilden sie einen natürlichen Abwehrmechanismus gegen Herbivore, z.B. Blattläuse, und durch Verringerung des QA-Gehalts in Lupinen wurde eine erhöhte Blattlausanfälligkeit beobachtet. Daher ist die Züchtung von Blattlaus-resistenten Sorten mit zugleich niedrigem QA-Gehalt erforderlich, um Ertragsverluste zu vermeiden und die Anwendung von Insektiziden zur Blattlausbekämpfung zu reduzieren.

Als Voraussetzung für eine solche Züchtung ist die Untersuchung eines Sortiments von schmalblättrigen Lupinen-Genotypen hinsichtlich ihrer Akzeptanz als Wirtspflanze für Blattläuse sowie hinsichtlich ihres QA-Gehalts und ihrer Zusammensetzung erforderlich. Daher wurde die Vermehrung von *Macrosiphum albifrons, Aphis fabae, Aphis craccivora, Acyrthosiphon pisum* und *Myzus persicae* als wahrscheinlich wichtigste schädigende Blattlausarten an 46 Genotypen der schmalblättrigen Lupine unter kontrollierten Bedingungen untersucht, und der QA-Gehalt und dessen Zusammensetzung analysiert. Die Ergebnisse zeigen, dass sich die Lupinenblattlaus (*M. albifrons*) unabhängig von hohem bzw. niedrigem QA-Gehalt an allen untersuchten Genotypen entwickeln kann. Bei *A. fabae, A. pisum, M. persicae* und *A. craccivora* wurde eine negative Korrelation zwischen der Blattlausvermehrung und dem QA-Gesamtgehalt festgestellt. Einige Genotypen konnten jedoch identifiziert werden, die einen geringen QA-Gesamtgehalt bei zugleich keiner oder nur deutlich reduzierter Blattlausvermehrung aufweisen. Es konnte gezeigt werden, dass die Blattvermehrung nicht nur durch den

Gesamtgehalt, sondern auch durch die Zusammensetzung der QAs beeinflusst wird, wobei für 13-Tigloyloxilupanine die größten Effekte nachgewiesen werden konnten.

Des Weiteren wurde der Einfluss des Lupinen-Genotyps auf das Saugverhalten einiger Blattlausarten, als Indikator für dessen Eignung als Wirtspflanze, analysiert, um die Auswirkungen der enthaltenen QAs zu untersuchen. Hierzu wurde das Saugverhalten der oben genannten Blattlausarten an vier Genotypen mit unterschiedlichem QA-Gehalt mittels „Electrical Penetration Graph" (EPG) beobachtet. Die Ergebnisse bestätigten die Beobachtungen der Vermehrungsversuche, da *A. fabae, A. craccivora, A. pisum* und *M. persicae* bei hohem QA-Gehalt eine verringerte Sauaktivität zeigten, wohingegen *M. albifrons* dadurch nicht negativ beeinflusst wurde. Die größten Unterschiede wurden hinsichtlich des Saugverhaltens am Phloem beobachtet. Bei Genotypen mit hohem Gehalt oder ungünstiger QA-Zusammensetzung wurde ein selteneres Auftreten sowie eine verkürzte Dauer der Saugphasen am Phloem nachgewiesen, mit Ausnahme von *M. albifrons*. Der in den Vermehrungsversuchen identifizierte Genotyp (cv. 'Kalya') mit niedrigem QA-Gesamtgehalt bei zugleich verminderter Blattlausanfälligkeit, zeigte auch einen Einfluss auf das Einstich- und Saugverhalten.

Die in den Versuchen identifizierten Genotypen mit einem niedrigen QA-Gesamtgehalt in Verbindung mit einer reduzierten Blattlausentwicklung können als Ausgangspunkt für die Züchtung QA-armer, schmalblättriger Lupinensorten mit Resistenz gegen Blattläuse dienen. Der Gehalt an 13-Tigloyloxilupanin konnte dabei als Merkmal identifiziert werden, welches zur indirekten Selektion in Züchtungsprogrammen der schmalblättrigen Lupine herangezogen werden kann.

1. Introduction

1.1. *Lupinus* spp.

The genus *Lupinus* L. is indigenous to the Mediterranean region (Old World species) as well as to the Americas (New World species) (Cowling et al., 1998; Adhikari et al., 2012) and comprises more than 400 species of which four are of agronomic interest: *Lupinus angustifolius* L. (narrow-leafed lupin; Figure 1.1-1), *Lupinus albus* L. (white lupin), *Lupinus luteus* L. (yellow lupin) as Old World species, and *Lupinus mutabilis* Sweet (Andean lupin) as New World species (Reinhard et al., 2006). The history of Lupin domestication has embraced more than 4000 years, tracing back to the archaeological discovery of seeds in tombs of Egyptian Pharaohs of the XII dynasty (>2000 years BC) (Zhukovsky, 1929; Kurlovich, 2002; Clements et al., 2005).

According to the GBIF Global Biodiversity Information Facility (2019) the genus *Lupinus* is taxonomically classified as part of the family of *Fabaceae* (Table 1.1-1). Lupin leaves are usually palmately, generally divided into five up to 28 leaflets, with only a few species known to carry single leaflets (Gresta et al., 2017). The flowers are shaped in dense or open whorls on an erect stem (Figure 1.1-1). They are formed by an upper standard, two lateral wings and a keel, which is formed by two fused petals. Fruits are developed as pod, each containing several hard-coated seeds. Referring to the summary of Kaess and Wink (1997) the chromosome numbers of *Lupinus* spp. range from 2n = 32, 36, 38, 40, 42, 50 and 52 in Old World lupins. For the broadly defined polymorphic group of New World lupins it is suggested that the base chromosomal number is x = 6, and they are regarded as paleopolyploids which behave as diploids (Dunn, 1984; Ainouche and Bayer, 1999). For most of them a chromosome number of 2n = 48 is reported, with occasional species having 2n = 36 or 96 (Ainouche and Bayer, 1999; CAMILLO et al., 2006; Office of the Gene Technology Regulator, 2013).

Table 1.1-1: Taxonomic classification of the genus *Lupinus* spp. (GBIF Global Biodiversity Information Facility, 2019)

Kingdom	Plantae
Phylum	Tracheophyta
Class	Magnoliopsida
Order	Fabales
Family	Fabaceae
Genus	Lupinus L.

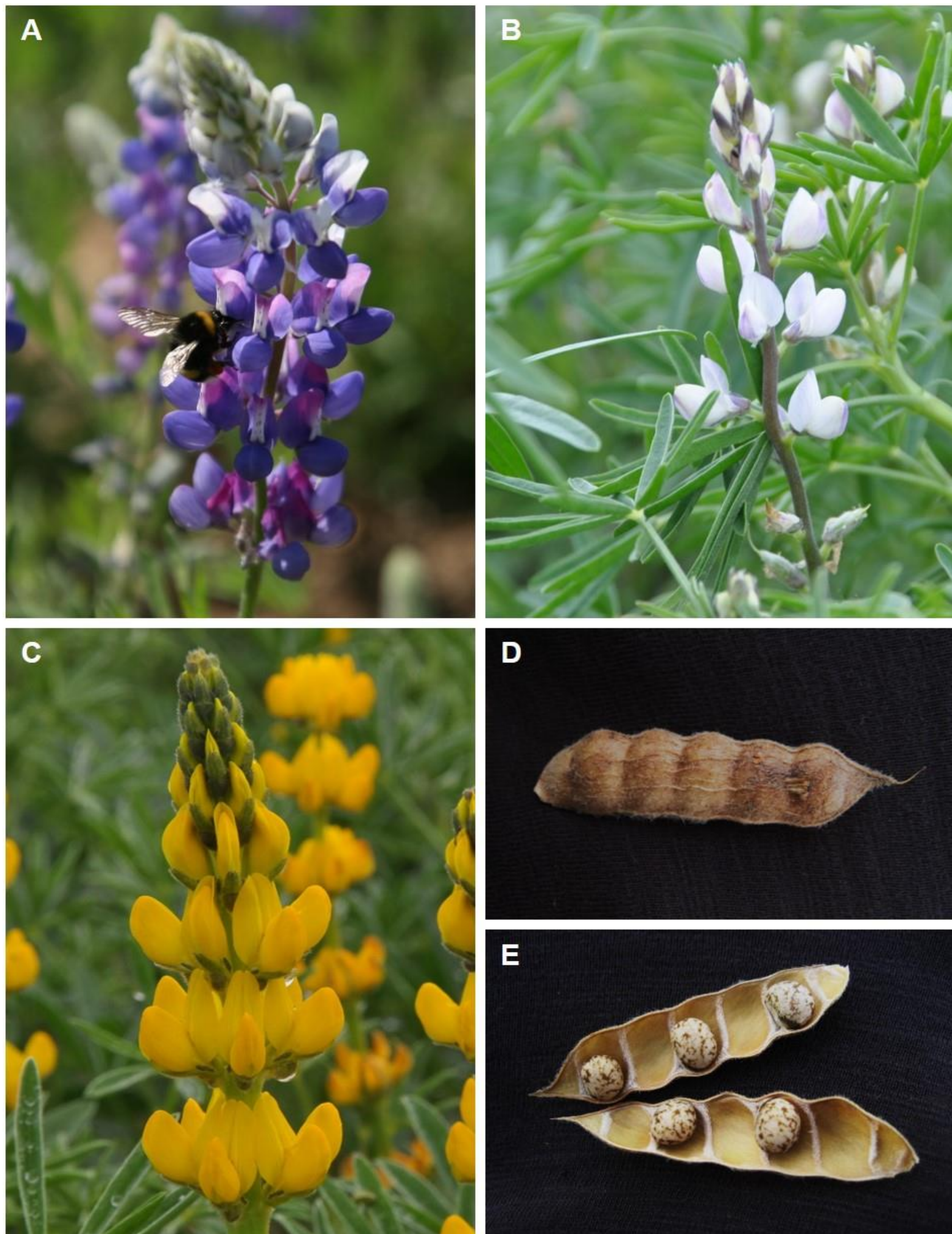

Figure 1.1-1: Example of flower phenology of commonly cultivated lupin species
A. Narrow-leafed lupin (*Lupinus angustifolius*), B. White lupin (*Lupinus albus*), C. Yellow lupin (*Lupinus luteus*), D. Pods and E. seeds of *L. angustifolius* (source: J. Philippi, E. Schliephake)

Due to many favourable agronomic features and their use as food and feed, lupin cultivation is of growing interest (Kordan et al., 2012). Especially the high protein content of 27-40% in lupin seeds is important for animal feed as well as for human nutrition,

whereby species of *L. luteus* and *L. albus* have a higher protein content than *L. angustifolius* (Jansen et al., 2013). Lupins have unique carbohydrate properties characterized by negligible levels of starch, high levels of soluble and insoluble non-starch polysaccharide (NSP), and high levels of raffinose oligosaccharides (van Barneveld, 1999). Their seeds are high in total dietary fibre (~40 g/100 g dry basis), making the lupin unique among other grains and legumes (Johnson et al., 2017).

Above and beyond the high protein content, lupins have the capability for nitrogen fixation. As well known for numerous plants belonging to the family of Fabaceae, lupins are able to take part in a mutualistic symbiosis with bacteria of the family Bradyrhizobiaceae, resulting in the capability to bind elementary nitrogen (N_2) and thus making it biologically available (Jarabo-Lorenzo et al., 2003; Stępkowski et al., 2005; Gresta et al., 2017). According to Zahran (1999) the biological N_2 fixation represents the major source of N input in agricultural soils, and the major N_2-fixing systems are the symbiotic systems [e.g. *Bradyrhizobium*-legume symbiosis], with capability of playing a significant role in improving the low-N soil fertility and productivity.

In addition lupins are also capable for organic phosphorus and micronutrients release from soil (Sujak et al., 2006), and have a high potential to mobilize nutrients for themselves and for interplanted or subsequent rotation crops (Johnson et al., 2017). According to Lambers et al. (2013) the phosphorus-acquisition strategy of *Lupinus* spp. is non-mycorrhizal or weakly mycorrhizal at most; instead they release vast amounts of phosphate-mobilizing carboxylates (inorganic anions) via their roots (*L. angustifolius* and *L. mutabilis* without specialized roots, *L. albus* with cluster roots, *L. luteus* with cluster-like roots), which makes lupines ideally suited for either impoverished soils or soils with large amounts of phosphorus that is poorly available for most plants, e.g. acidic or alkaline soils. Furthermore, it is described that the deep tap root of *L. angustifolius* allows to effectively extract and recycle potassium (Rowland et al., 1986; Johnson et al., 2017). These characteristics are of special interest in organic farming where utilization of chemical fertilizer is impossible (Jensen et al., 2004), and therefore the use of lupins as green manure is of agronomic importance.

Due to the well-developed tap root system of lupins, providing sustenance with water and nutrients from deeper soil layers, they often endure drought periods quite well. Hence, in addition to the nutrient fixation, lupins may also increase the access to water resources for the subsequent crop (Henderson 1989) which generates a high potential for sustainable crop rotation systems (Jensen et al. 2004). Furthermore, for the subsequent cereal crops a better weed competition and provision of a ‘disease break” is

reported, leading to increased yield of the next crop in the rotation (Asseng et al., 1998; Seymour et al., 2012; Johnson et al., 2017).

The fungal disease anthracnose [*Colletotrichum lupini* (Bondar) Nirenberg, Feiler & Hagedorn (Damm et al., 2012)] is a severe disease of lupins, occurring in all lupin-growing countries (Adhikari et al., 2011). Especially the production of yellow lupin was highly affected by high yield losses in traditional lupin-growing countries such as Poland, Portugal, Russia, France, and Germany due to unavailability of resistant cultivars. Thus, the lupin breeding programmes in these countries have focussed on narrow-leafed lupin because of the availability of anthracnose-resistant cultivars in this species (Gresta et al., 2017), and hence the narrow- leafed lupin (*L. angustifolius*) has gained more importance.

1.2. Lupin growing area and production quantity worldwide

Lupinus angustifolius L., *L. albus* L., *L. luteus* L. and *L. mutabilis* Sweet are agronomically important species (Reinhard et al., 2006), and growing of *Lupinus* spp. is widespread. According to the FAO – Food and Agriculture Organization of the United Nations (FAOSTAT, 2018) in 2012 to 2016 an overall average area harvested of 862654 ha/year corresponding to 1148158 t/year was recorded. The main production in 2012 to 2016 arose from Oceania (Australia), followed by Europe (mainly Poland, The Russian Federation and Germany), Africa (mainly Morocco) and the Americas (mainly Chile and Peru), while the production in Asia is vanishingly small (Table 1.2-1 and Table 1.2-2).

In Europe *L. angustifolius*, *L. luteus* and *L. albus* are cultivated. In Germany, *L. angustifolius* is the main species grown, while in Poland both *L. angustifolius* and *L. luteus* are cultivated, and *L. albus* is mainly cultivated in the south, mainly in Italy, France and Spain (Gresta et al., 2017).

Table 1.2-1: Average area harvested and production quantities in the world and its regions during 2012-2016 (FAOSTAT, 2018)

Region	Area harvested		Production	
	ha/year	%	Tonnes/year	%
Oceania	500770	58.05	653372	56.91
Europe	237688	27.55	384828	33.52
Africa	94818	10.99	66721	5.81
Americas	29313	3.40	43102	3.75
Asia	65	0.01	135	0.01
World	**862654**	**-**	**1148158**	**-**

Table 1.2-2: Average area harvested and production quantities during 2012-2016 by country (FAOSTAT, 2018)

Country	Area harvested [ha/year]	Production [t/year]	Country	Area harvested [ha/year]	Production [t/year]
Australia	500770	653372	**Spain**	4712	2738
Poland	106282	163415	**Ecuador**	3762	1355
Russian Federa.	59910	99845	**Egypt**	496	1027
Morocco	83946	56143	**Greece**	417	703
Germany	22980	38340	**Slovakia**	301	403
Ukraine	17040	28186	**Austria**	118	228
Chile	15419	28933	**Switzerland**	82	226
Belarus	13109	28910	**Latvia**	132	166
Peru	10025	12654	**Argentina**	107	161
South Africa	10376	9551	**Hungary**	124	108
France	5105	12543	**Lebanon**	57	127
Lithuania	4004	4282	**Syrian Arab Rep.**	8	8
Italy	3366	4728	**Portugal**	7	7

1.3. Quinolizidine Alkaloids and breeding of “sweet lupins”

Wild lupins produce a high level of quinolizidine alkaloids. These are toxic secondary metabolites derived from the amino acid lysine (Frick et al. (2017); Figure 1.3-1), which protect them from herbivores (Wink, 1998; Ridsdill-Smith et al., 2004; Adhikari et al., 2012). Quinolizidine alkaloids are produced in leaf chloroplasts, then translocated all over the plant via the phloem and stored in epidermal cells and in seeds (Wink and Witte, 1984; Wink et al., 1995; Wink, 1998). Lee et al. (2007) reported, that the QA concentration increases towards the plant apex in newly produced tissues, particularly in reproductive organs, and levels of QA are 10–30 times lower in xylem than in phloem exudates. QAs synthesized in leaves can account for up to 8% of their dry weight (Gresta et al., 2017).

QA content and composition in leaves of narrow-leafed lupins can be determined by gas chromatography and subsequent mass spectrometry (GC/MS), and data and Kovats retention indices published by Wink et al. (1995) can be used for identification of QAs. A detailed description of the analytical method is given in the underlying publications of this thesis (Philippi et al., 2015, 2016).

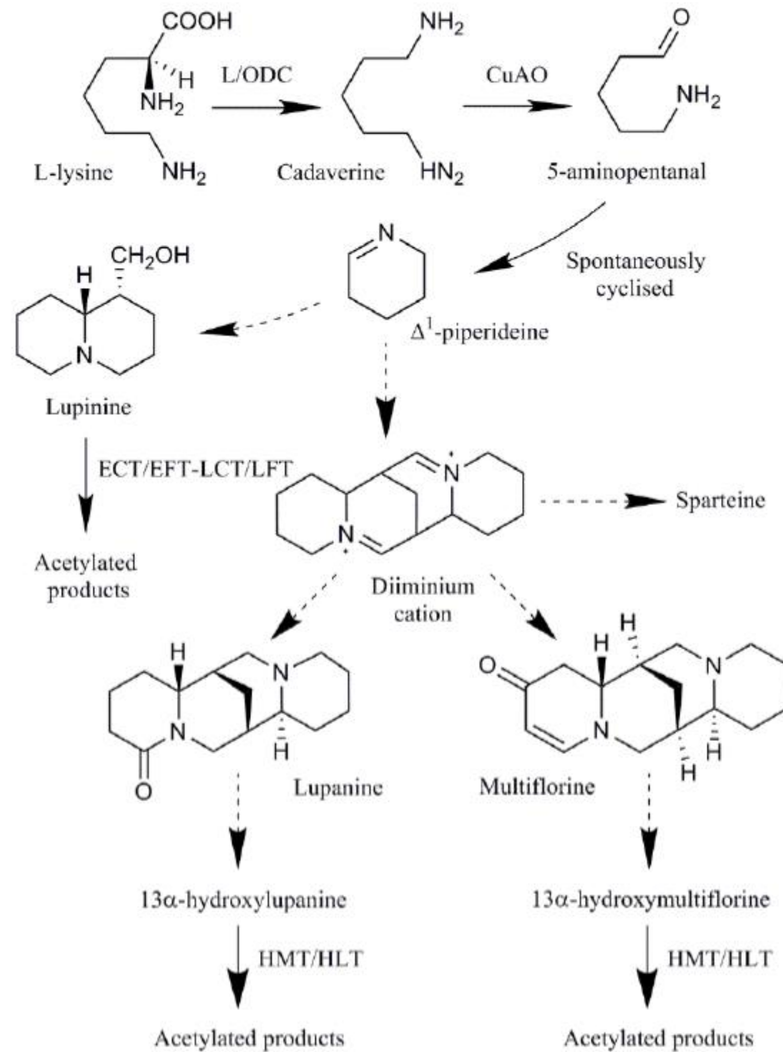

Figure 1.3-1: Quinolizidin alkaloid biosynthetic pathway (Frick et al., 2017) (Dotted lines represent unknown reactions. L/ODC=lysine decarboxylase, CuAO=copper amine oxidase, ECT/EFT-LCT/LFT=ρ-coumaroyl-CoA/ feruloyl-CoA: (+)-epilupinine/(–)-lupinine O-coumaroyl/ feruloyltransferase, HMT/HLT=tigloyl-CoA:(–)-13α-hydroxymultiflorine/ (+)-13α-hydroxylupanine O-tigloyltransferase)

Alkaloids of the quinolizidine group are considered to be the main anti-nutritional substances in lupins because of their bitter taste and their toxicity for animals and humans, causing convulsions, trembling, and death from respiratory and cardial arrest (Michael, 2002, 2003; Sujak et al., 2006; Ganzera et al., 2010). The level of QAs in grains can vary under field conditions from year to year due to environmental conditions (Cowling and Tarr, 2004).

Due to the growing interest based on the above-mentioned favourable characteristics of lupins, breeding for agronomically important traits such as a low alkaloid content was initiated. As alkaloids of the quinolizidine group are considered to be the main anti-

nutritional substances in lupins because of their bitter taste and toxicity (Michael, 2002, 2003; Sujak et al., 2006), breeding for so called sweet lupins with a markedly reduced QA content of <0.05% in seeds (Sengbusch, 1931; Fischer and Sengbusch, 1935; Sengbusch, 1942) was a milestone to harness lupins as food and feed crops. A threshold of 0.05% (500 mg/kg) for animal feed and 0.02% (200 mg/kg) for human nutrition is the currently acceptable level for lupins classified as “sweet” (Jansen et al., 2009), which is approximately 100 fold lower than wild type seed alkaloid levels (Johnson et al., 2017).
Several low-alkaloid mutations are known for narrow-leafed lupins, such as the natural mutations *iucundus, esculentus* (Hackbarth and Sengbusch, 1934), and *depressus* (Hackbarth and Troll, 1956; Schwarze and Hackbarth, 1957), and an x-ray induced mutation which led to the identification of the locus *tantalus* (Zachow, 1967). Markers linked to the low alkaloid locus *iucundus* were first developed by Li et al. (2011). In 2019 Kroc et al. (2019b) identified candidate genes linked to this locus, and for one out of these (APETALA2/ethylene response transcription factor RAP2-7) the co-dominant derived cleaved amplified polymorphic sequence (dCAPS) marker iuc_RAP2-7 was developed (Kroc et al., 2019a) which can be used for marker-assisted selection and therefore accelerated breeding of low-alkaloid cultivars.

1.4. Aphids

According to Blackman and Eastop (2019) the known world aphid fauna (Table 1.4-1) consists of a total of about 5000 species, found on about 300 plant families of all kinds, such as herbaceous, woody or shrubby plants or trees. Aphids are distributed worldwide, although predominantly occurring in the northern temperate zone, with only few species in the tropics.

Damage of plants results either directly by feeding, i.e.by removal of sap or wounding of tissue, or in at least some cases for trees by the toxic effect of saliva, or indirectly by transmitting viruses (Blackman and Eastop, 2019).

Table 1.4-1: Taxonomic classification of the family Aphididae (GBIF Global Biodiversity Information Facility, 2019)

Kingdom	Animalia
Phylum	Arthropoda
Class	Insecta
Order	Hemiptera
Family	Aphididae

Aphids can be grouped as monophagous insects, feeding on one plant species or genus only, as oligophagous, feeding on plants of one plant family, or as polyphagous, feeding and reproduction on several plant species out of different plant families. The life cycle of aphids is divers, differing between genera and even species, and three possible life-cycle mechanisms are distinguished (Börner, 2009):

1. Heteroecious holocyclic: Complete life cycle with alternation between sexual reproduction and parthenogenesis with alternation from primary to secondary host

An example for heteroecious holocyclic development is *Aphis fabae*. As described by Blackman and Eastop (2019) (Figure 1.4-1) the fundatrix (1) develops from an overwintering egg and founds a colony (by parthenogenesis) on new growth of the primary host spindle (*Euonymus europaeus)* or guelder rose (*Viburnum opulus*) in spring, giving birth by viviparity. In May-June emigrant alatae (2) are developed, migrating to various herbaceous secondary hosts through spring and summer (3) where succeeding generations are developed. When colonies become larger, alate viviparae are produced which migrate to other plants for colony founding. Influenced by decreasing daylength in autumn, colonies on secondary hosts produce alate gynoparae (4) and alate males (5), which migrate back to the primary host. The alate gynoparae give birth to the oviparae (6) (apterous sexual females), which mature on primary host leaves just before they fall. They mate with alate males, and lay fertilized overwintering eggs (7) in bud axils.

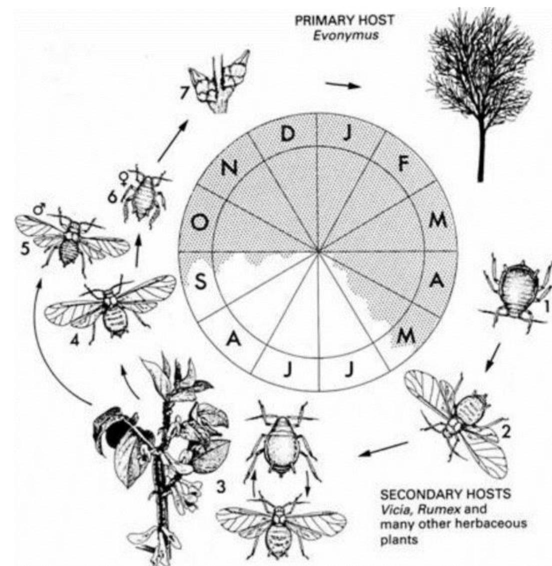

Figure 1.4-1: Heteroecious holocyclic life cycle of *A. fabae* (Blackman and Eastop, 2019)

2. Monoecious holocyclic: Complete life cycle with alternation between sexual reproduction and parthenogenesis without host-alternation

The fundatrix develops from an overwintering egg and founds a colony on new growth of the host in spring by parthenogenesis. When colonies become larger and more crowded, alate viviparae migrate to plants of the same host or on a small range of closely-related host plants for founding new colonies. At the end of the vegetation period, oviparae (apterous sexual females) and alate males develop. They mate and the oviparae lay fertilized overwintering eggs.

3. Anholocyclic: "incomplete" life cycle without sexual but only parthenogenetical reproduction.

Anholocyclic development is mainly known for aphids in worm climates, as there is no need to produce frost-resistant winter eggs, resulting in the loss of the sexual part of the life cycle and reproduction solely by parthenogenesis (Börner, 2009). Some species are entirely anholocyclic and have no known sexual morphs, while others (e.g. *Myzus persicae*) may be anholocyclic in warmer regions and holocyclic under cold temperate climate (Blackman and Eastop, 2019).

1.5. Aphids on *Lupinus angustifolis*

As listed in Holman (2009) several aphid species are known to infest lupins, and aphid infestation may cause yield losses up to 100% by feeding (Kordan et al., 2008). Furthermore, narrow-leafed lupins are susceptible to the *Bean Yellow Mosaic Virus* and *Cucumber Mosaic Virus*, both transmitted by aphids (Garlinge, 2005). Budding and flowering is the period of the highest vulnerability of lupins to aphid infestation, due to the fact that severe feeding damage on growing tips can cause dropping of buds, abortion of flowers and reduction of pod set (Micic and Thomas, 2018).

According to the host list of Blackman and Eastop (2019) *L. angustifolius* is known as host plant for the following aphid species (among others):

Lupin aphid - *Macrosiphum albifrons* (Essig)

The apterous morph *of M. albifrons* (Figure 1.5-1) is pale bluish-green, dusted with white wax (Fritzsche and Keilbach, 1994), with a body length of 3.2-5.1 mm (adults) and an oval shape. The siphunculi are light brown with dark tips, have about 0.21-0.32 times the body length, and about 1.6-2.2 times the length of the cauda. The cauda is pale and slender without any constrictions, and antennae and legs are pale or dusky with blackish apices. (Blackman and

Figure 1.5-1: ***M. albifrons*** **(© J. Philippi)**

Eastop, 2019). Alatae have a dusky head, brown pterothorax, a bluish green abdomen with small marginal spots and dusky siphunculi.

M. albifrons originates in North America where sexual forms develop in autumn, and the aphid has the ability to overwinter as eggs (Müller et al., 1990). It is feeding monophagous on lupin species and spends its entire life cycle on this crop. In 1981 it appeared in Europe (United Kingdom) for the first time (Gruppe and Roemer, 1988) and is now widely distributed and considered an invasive pest species over much of Europe. Experimental tests of exposure to freezing conditions in the United Kingdom are reported by Carter and Nichols (1989), indicating that *M. albifrons* probably survives and reproduces on lupin species through most European winters in the parthenogenetic viviparous stage, due to its low-temperature tolerance.

Cowpea aphid - *Aphis craccivora (*Koch)

The apterous morph of *A. craccivora* (Figure 1.5-2) is black-greenish, showing on the dorsal abdomen a big shiny blackish spot (Fritzsche and Keilbach, 1994). Immatures are lightly dusted with wax (Blackman and Eastop, 2019)

Figure 1.5-2: ***A. craccivora*** **(© J. Philippi)**

It is 1.2-2-3 mm long and has a rounded-oval shape. The siphunculi and cauda are black, the antennae have a length of more than half the body length (Fritzsche and Keilbach, 1994).

A. craccivora occurs on the young growth of numerous plants, particularly of fabaceae where it is a major pest, while plants in other families tend to be colonised more in the dry season (Blackman and Eastop, 2019). It occurs worldwide, but is mainly common in warm temperate and tropical regions. It lives anholocyclic almost everywhere, but cases of a sexual phase with alate males have been reported from Germany, India and Argentina (Blackman and Eastop, 2019). In areas with cold winters *A. craccivora* overwinters e.g. on alfalfa*,* alternating in spring to other host plants out of several plant families, although preferring fabaceae. It is known as vector of several viruses.

Aphis craccivora is known to be usually ant-attended (Blackman and Eastop, 2019). It often occurs in dense colonies on a single plant before moving to adjacent plants, and is known for a more patchy distribution in the lupin crop (Micic, 2018).

Black bean aphid - *Aphis fabae* (Scopoli)

Aphis fabae (Figure 1.5-3) apterae are dark grey-greenish to (dull) black, with a rounded-oval shape (Fritzsche and Keilbach, 1994). The siphunculi and cauda are black, the antennae are shorter than 4/5 of the body length, which is about 1.5-3.1 mm (Blackman and Eastop, 2019).

A. fabae overwinters as winter eggs on spindle (*Euonymus europaeus)* and on guelder rose (*Viburnum opulus)* as primary host, alternating in spring to secondary host plants out of several plant families (Börner, 2009), including the young growth of some trees, and many other crops (Blackman and Eastop, 2019). In autumn, oviparae on the primary hosts are small and the males are alate. According to Blackman and Eastop (2019) there seems to be a complex of sibling species or partially interfertile subspecies of *A. fabae* in Europe, and anholocyclic populations of aphids of this *A. fabae* group (especially the closely related *A. solanella*) occur on secondary hosts e.g. in southern Europe. *Aphis fabae* is common in the northern hemisphere, and occurs in many tropical and subtropical countries. *Aphis fabae* is known to be usually ant-attended (Blackman and Eastop, 2019).

Figure 1.5-3: *A. fabae* (© J. Philippi)

Pea aphid - *Acyrthosiphon pisum* (Harris)

Acyrthosiphon pisum apterae (Figure 1.5-4) are pink or pale green to yellow, depending the race, with red eyes (Fritzsche and Keilbach, 1994). The body size ranges from 2.5 to 4.4 (-5.5) mm. Cauda and siphunculi are very long and pale. The antennae are longer than the body, and are dark at apices of segments, often lightly wax-dusted (Blackman and Eastop, 2019).

Figure 1.5-4: *A.n pisum* (© J. Philippi)

A. pisum is monoecious holocyclic in temperate regions, and according to Frantz et al. (2010) apterous and alate males are produced in differing proportions, depending on the host. Overwintering as winter eggs occurs mainly on perennial *Vicia* spp., and in rarer

cases on *Trifolium pratense* (Thieme and Heimbach, 1996). *A. pisum* occurs on the young growth and developing pods of many herbaceous and some shrubby or woody Fabaceae as secondary host and is widespread all over the world (Blackman and Eastop, 2019). The pea aphid is known as vector for more than 30 non-persistent and persistent viruses (Blackman and Eastop, 2007), e.g. the *Pea enation mosaic virus* (Börner, 2009).

Green peach aphid - *Myzus persicae* (Sulzer)

Myzus persicae apterae (Figure 1.5-5) are greenish-yellow to green, longish-egg shaped and 1.0 to 2.3 mm long (Fritzsche and Keilbach, 1994). The antennae are in most cases about the size of the body length.

Figure 1.5-5: *Myzus persicae* (© J. Philippi)

The fundatrix and her progeny have tapering, unswollen siphunculi, which are of about the double length of the cauda (Blackman and Eastop, 2019).Immature alatae are often pink or red, especially in autumn populations (and immature males are yellow) and mature alatae have a dark dorsal abdominal patch (Blackman and Eastop, 2019).

Both, holocyclic and anholocyclic forms are known for *M. persicae*. It overwinters either holocyclic on hosts of mainly *Prunus* spp. (*Prunus persica* or in north-eastern North America on *P. nigra* (Shands et al., 1969)), migrating in spring to secondary host plants in over 40 different plant families (Blackman and Eastop, 2019) or anholocyclic as imago on sheltered places (Börner, 2009). In milder climates, populations are partially anholocyclic on the secondary host plants, and in tropics or in the absence of the primary host only the anholocyclic form is present (Blackman and Eastop, 2019).

M. persicae is probably of East Asian origin (Blackman and Eastop, 2019), but is now the most abundant aphid worldwide and is the most important virus vector in dicotyledonous plants. As a polyphagous, the green peach aphid is feeding on more than 400 dicotyledonous plants out of more than 40 plant families and has the potential to infest sweet lupins at a high rate (Edwards, 2001; Börner, 2009). According to Micic (2018), *M. persicae* tends to be evenly distributed throughout the lupin crop.

1.6. Electrical Penetration Graph (EPG) - Investigation of feeding behaviour

Aphids are causing damages on host plants either directly by feeding and deprivation of assimilates with their stylet (sucking-piercing mouthpart structures) or by acting as vectors for the transfer of plant viruses during feeding (Valenzuela and Hoffmann, 2015).

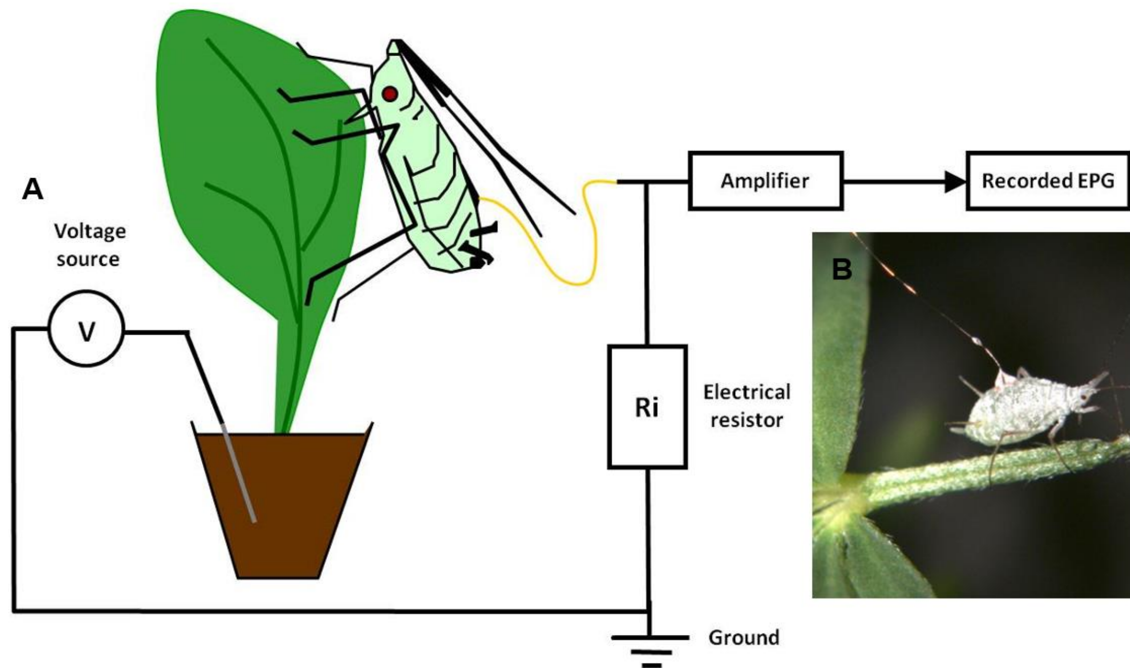

Figure 1.6-1: A) Scheme of Electrical Penetration Graph (EPG) and B) Lupin aphid attached to gold wire.

Aphid feeding behaviour can be recorded by using the so called electrical penetration graph – EPG (Tjallingii, 1978, 1994). Insect and plant are made part of an electrical circuit (Figure 1.6-1; source: http://www.epgsystems.eu, modified), including a low voltage source, an input resistor and an amplifier (Tjallingii (2006). The aphid is connected to an electrode, which is a thin, flexible gold wire, attached on the aphid's dorsum by using conductive, water-based silver glue. The plant electrode is inserted into the potting soil of the plant. When aphid stylets are inserted in the plant tissue for probing the electrical circuit is completed. The plant voltage is adjusted so that when the stylet tips are inserted intercellularly the signal voltage is positive and when the tips are intracellularly the signal voltage is (mostly) negative.

As described by Salvador-Recatalà and Tjallingii (2015) with the EPG system the electromotive force originated potentials generated in the plant tissue or the insect in addition to the potentials arising from resistance in the insect can be reported. This provides biological relevant information on events during plant penetration by aphids.

The signals shown for probing (stylet penetration) and non-probing phases can be clearly distinguished (exemplarily displayed in Figure 1.6-2). Within the probing, the pathway phase, xylem phase, phloem phase and phase of derailed stylet mechanics (indicating difficulties during penetration process) can be observed, each containing one or more patterns of voltage fluctuation, called waveforms (Tjallingii, 2006). By experimental investigation these waveforms were allocated to specific probing activities of the insects and as such to locations in the plant tissue of the stylet tips (Tjallingii, 1978, 1985; Tjallingii and Esch, 1993; Tjallingii, 1994, 2006).

Aphids feeding starts with penetrating the cuticula with their stylet into the epidermis, starting at the border between two cells, and with secretion of sheath material (Tjallingii, 1994). With their stylets, aphids penetrate plant tissues by probing intercellularly through epidermal and mesophyll cell layers (Gao et al., 2008) while continuously excreting gelling saliva (building the salivary sheath) during the pathway phase (Tjallingii, 2006). Short term intracellular pathway puncturing events, so called potential drops, can be reported along the stylet pathway (Tjallingii, 1994). Ultimately aphids feed specifically from the phloem sieve element where they may have a long-lasting association with their host (Gao et al., 2008), depending on the acceptance as host plant. Virus inoculation by salivation in the plant cell and virus acquisition by ingestion can either occur during intracellular puncturing (potential drop; non-persistent viruses) or during phloem feeding (persistent viruses). In addition, active xylem ingestion can be observed during feeding recording (Tjallingii, 1994), which is associated to water acquisition.

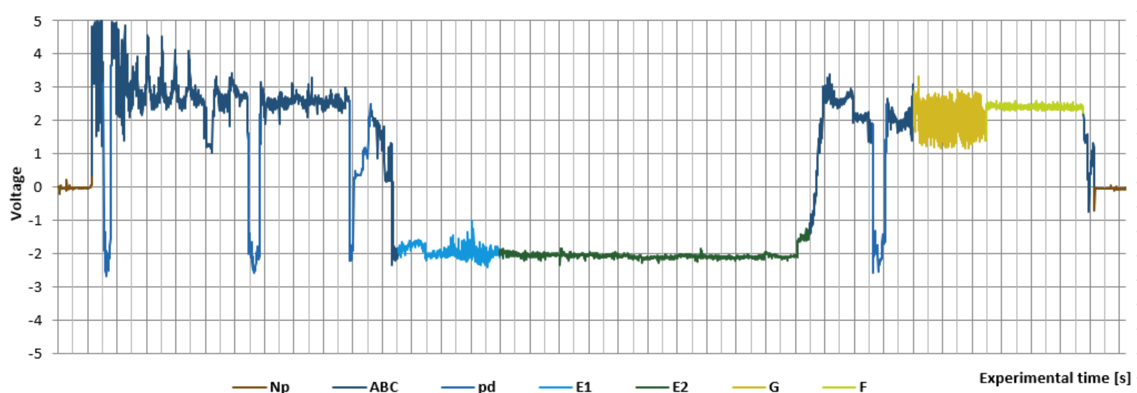

Figure 1.6-2: Exemplary scheme of EPG waveforms
Nonprobing periods (Np), extracellular pathway phase (C), potential drop (pd; stylet is puncturing the cell), phloem salivation phase (E1; release of saliva in the phloem), phloem feeding phase (E2; ingestion of phloem sap), xylem feeding phase (G; ingestion from xylem), derailed stylet mechanics (F; difficulties during penetration process; no ingestion)

Evaluation of a conclusive number of runs conducted with different aphid-genotype combinations, with regard to duration or number of probing phases or the time to e.g. the first occurrence of a specific phase, can provide information about the feeding behaviour and differences between the aphid-genotype combinations investigated. It can be used as an indicator to distinguish between aphid acceptance or denial of a genotype as host-plant.

1.7. Impact of the QA content on aphid susceptibility of lupins

It is described by Smith (2010) that in the Jurassic (~200 million years ago) a shift of arthropod feeding from polyphagous to specialized oligophagous feeding and subsequently monophagy occurred and at a similar point in the fossil record occurrence of plant alkaloids was detected, which suggests that plants began to actively evolve these as defence compounds. Fabaceae are able to fix atmospheric nitrogen via symbiosis, thus nitrogen for production of secondary metabolites was easily available and Wink and Mohamed (2003) concluded, that it is not surprising that nitrogen-containing secondary metabolites such as QAs are common to Fabaceae.

Wild *Lupinus* species produce a high level of quinolizidine alkaloids to protect themselves from herbivores (Wink, 1998). Only very specialized species such as the lupin aphid, *Macrosiphum albifrons* Essig (Hemiptera: Aphididae), are able to use lupins as host plants and to feed from high QA containing plants. It was observed that *M. albifrons* is able to store QAs when feeding on QA-rich lupins, and to use these for defence against predators such as the carabid *Carabus problematicus* Herbst or the sevenspotted lady beetle *Coccinella septempunctata* L. (Wink and Roemer, 1986; Gruppe and Roemer, 1988; Emrich and Wink, 1992).

Wink & Roemer (1986) concluded that QAs serve the lupin aphid as a cue to find suitable host plants, whereas plants with low QA content seem to be less attractive. Furthermore, Finlayson et al. (2010) found that all four studied coccinellids species consumed fewer *M. albifrons* compared with three other aphid species, likely because of deterrent compounds sequestered by this species from its host plant.

As *M. albifrons* spends its whole life cycle on *Lupinus* spp. it is well-adapted, in particular to those lupins with a high QA content, and thus it is able to cause considerable infestations and concomitant yield losses (Ferguson, 1994). However, for other aphids a high content of QAs in *Lupinus* spp. seems to be an almost insuperable barrier for

considerable population development. Associated with breeding of the so called sweet lupins with a reduced QA content to harness lupins in human food and animal feed production, an increase of susceptibility to insect herbivores and plant pathogens was observed (Wink, 1985, 1988; Wink and Witte, 1991; Reinhard et al., 2006; Michael, 2008; Ganzera et al., 2010)

Most divers animals, such as leaf miners (Agromyzidae) or rabbits refuse feeding on QA-rich lupins, while "alkaloid-free", sweet cultivars are accepted for feeding (Wink, 1988; Wink and Mohamed, 2003). Denial of high QA content is most likely based on the toxic effects on the nervous system of animals, affecting mainly nicotinic and muscarinic acetylcholine receptors and inhibiting Na+ and K+ channels (Wink et al., 1998; Wink and Mohamed, 2003).

In Australia as one of the major lupin-growing areas, aphids are regarded as important pests, which cause severe yield losses by feeding (Berlandier and Sweetingham, 2003) and virus transmission (Thackray et al., 2004; Valenzuela and Hoffmann, 2015). Severe infestation of sweet lupins with aphids may cause yield losses up to 100% by feeding (Zehnder et al., 2001; Kordan et al., 2008).

It was reported by French (2004) that differences in the susceptibility for aphid feeding and its resulting damage are known for Australian narrow-leafed lupin cultivars. Wink (1992) showed that e.g. *Myzus persicae* as generalists only fed on "sweet" lupins but never on QA-rich varieties with high QA content in the phloem. The potential of *M. persicae* to infest sweet lupins at a high rate was also reported by Edwards (2001), indicated by high growth rate and high rate of survivorship on the sweet cultivar 'Tallerack'. Moreover, Berlandier (1996) concluded that the QA level suppresses the fecundity of *M. persicae.*

Furthermore, findings of Berlandier and Sweetingham (2003) showed in field trials in Western Australia that the number of aphids of the cowpea aphid (*Aphis craccivora*), the green peach aphid (*Myzus persicae*) and the blue-green aphid (*Acyrthosiphon kondoi*) was depending on the cultivar of the narrow-leafed and yellow lupin and thus they have the potential to cause substantial yield losses in cultivars with extensive aphid colonization. It is reported that *A. craccivora* is ubiquitous in Australian lupin-growing areas, causing severe yield losses by direct feeding on vegetative and reproductive parts of sweet lupins (Zehnder et al., 2001). A severe colonisation of lupin crops with *A. craccivora* was shown to cause rapid wilting (Micic, 2018).

On lupins with a reduced QA content the pea aphid *A. pisum* is of importance, but it is not able to colonize QA-rich lupins (Gruppe and Roemer, 1988; Kordan et al., 2008; Kordan et al., 2012). It was reported by Dreyer et al. (1985) that some lupin QAs investigated in diet bioassays inhibit feeding by the pea aphid. In addition, the generalist *A. fabae* can be observed on sweet lupins, but it is unable to colonize lupins with high QA content (Gruppe and Roemer, 1988; Ferguson, 1994).

These findings show the potential of damages by aphid feeding in sweet cultivars of narrow-leafed lupins, resulting in severe yield losses. Thus, depending on the cultivar, spraying of insecticides is indicated (Garlinge, 2005) which is expensive and harmful to the environment.

1.8. Objectives

Breeding of lupin varieties with a low quinolizidine alkaloid content has improved the usability of lupins. Due to their nutrient composition and a higher protein content than other native protein plants, lupins are usable in a versatile way and their suitability for cultivation in domestic areas enables the reduction of import of vegetable protein, i.e. soy bean protein. In addition, lupins improve the soil structure and promote natural nitrogen retention in the soil, thus they can be a valuable part of a crop rotation program.

An important aspect of the usability of lupins as food and feed is the reduction in the content of QAs, which in addition to the bitter taste also have a toxic effect on various organisms. By their reduction within the breeding process, the natural defence against various pathogens, i.e. aphids, is reduced. Aphids damage the plants through the deprivation of nutrients and are also acting as virus vectors, which is causing further harm. The selection of aphid-resistant lupin genotypes with at the same time low QA content in the seeds is therefore an important step to encourage an expansion of the acreage of lupins.

Taking into account the predicted climate change, an increased occurrence of aphids is expected. Rising temperatures and longer growing seasons increase the generation number of aphids and favour a quick adaptation to new hosts and a permanent establishment as a pest. An aphid infestation can be prevented by insecticides; however, its use is limited especially in organic farming, and occurrence of aphid resistance to pesticides can be observed. Moreover, for widely used insecticidal active substances out of the group of neonicotinoids (i.e. imidacloprid, clothianidin and thiamethoxam) a risk for bees was identified in assessments of the European authorities during the past years

(European Commission, 2019), which led to severe use restrictions for the respective active substances. The use of imidacloprid was limited to permanent greenhouses only and the applications for renewal of approval for the other two active substances were withdrawn, resulting in a reduced number of available approved active substances for control of insects. Furthermore, it is the declared aim of e.g. the European Union and the participating member states to generally reduce the amount of plant protection products released to the environment, as indicated in the Sustainable Use Directive (European Union, 2009) and the National action Plans of the member states. The selection of aphid-resistant genotypes thus represents an important target in breeding of lupins, in particular for the narrow-leafed lupin (*Lupinus angustifolius* L.) on which this thesis is focused.

With regard to these aspects, the aim of this thesis was to identify genotypes of the narrow-leafed lupin with resistance or low susceptibility against various aphid species, while simultaneously containing a low total QA content. Therefore, the content of different QAs in genotypes of the narrow-leafed lupin was investigated, and

(i) the correlation between the composition of the quinolizidine alkaloids and the resistance or susceptibility of 46 genotypes of *L. angustifolius* for infestation with *Aphis fabae, Aphis. craccivora, Acyrthosiphon pisum, Myzus persicae* and *Macrosiphum albifrons* was evaluated, to identify promising genotypes for breeding.

The feeding behaviour of above-mentioned aphid species on four genotypes of *L. angustifolius* with different QA content and composition was analysed, to investigate its direct impact on the aphids.

2. Correlation of the alkaloid content and composition of narrow-leafed lupins (*Lupinus angustifolius* L.) to aphid susceptibility.

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2.1. Abstract

Breeding of narrow-leafed lupins (*Lupinus angustifolius* L.) with a low alkaloid content, so called sweet lupins, increased the use for food and feed. Coincidentally the reduced alkaloid content increased the susceptibility for aphid infestation. Hence, breeding of resistant cultivars is needed to prevent yield losses and reduce the application of insecticides. As a prerequisite for this the evaluation of a diverse collection of narrow-leafed lupin genotypes is needed. Therefore, the multiplication of different aphid species, i.e. *Macrosiphum albifrons, Aphis fabae, Aphis craccivora, Acyrthosiphon pisum* and *Myzus persicae* (all Hemiptera: Aphididae) was investigated on 46 narrow-leafed lupin genotypes under controlled conditions. Furthermore, the alkaloid composition and total content of these genotypes was analyzed, in order to get information on their influence on the susceptibility to different aphid species. Results indicated that the multiplication of the well-adapted lupin aphid (*M. albifrons*) is not affected by the alkaloid content. In contrast, *A. fabae, A. pisum, M. persicae* and *A. craccivora* showed a negative correlation between aphid multiplication and alkaloid content (r = -0.493 to -0.350). However, several genotypes with a low total alkaloid content, e.g. Kalya, Bora and Borlu, were detected on which no or only a very limited aphid multiplication was observed, indicating that not only the total content, but also the alkaloid composition is influencing aphid development. By multiple linear regression analysis it turned out that especially 13-hydroxylupanine and 13-tigloyloxylupanine are involved in the reduced aphid

multiplication rate. Respective genotypes may be the starting point for diminishing aphid susceptibility in sweet lupins.

2.2. Introduction

Lupinus angustifolius L. (narrow-leafed lupin), *Lupinus albus* L. (white lupin), *Lupinus luteus* L. (yellow lupin) and *Lupinus mutabilis* Sweet (Andean lupin) are agronomically important species (Reinhard et al., 2006) which were grown in 2013 on 650.629 ha resulting in the production of 785.596 tons (FAOSTAT, 2015). Due to many favorable agronomic features and their use as food and feed, lupin cultivation is of growing interest (Kordan et al., 2012). Above and beyond the high protein content up to 27-40% (Jansen et al., 2013), lupins have the capability for nitrogen fixation and organic phosphorus release from soil (Sujak et al., 2006) which is of special interest in organic farming where the utilization of chemical fertilizer is impossible (Jensen et al., 2004). In addition to the nutrient fixation, lupins develop a deep taproot system which may increase the access to water resources for the subsequent crop (Henderson, 1989), generating a high potential for sustainable crop rotation systems (Jensen et al., 2004). However, wild lupins are producing a high level of quinolizidine alkaloids in leaf chloroplasts, which are distributed all over the plant via the phloem and stored in epidermal cells and in seeds (Wink and Witte, 1984; Wink et al., 1995; Wink, 1998). Such alkaloids act as a defense against insects and other herbivores (Reinhard et al., 2006; Michael, 2008; Ganzera et al., 2010) except for very specialized and well adapted species like the lupin aphid (*Macrosiphum albifrons*). Quinolizidine alkaloids bind to acetylcholine muscarinic (mAChR; e.g. angustifoline) or nicotinic acetylcholine receptors (nAChR; e.g. lupanine) (Wink, 2000). This mode of action is similar to the well described insecticidal pyridine alkaloid nicotine and neonicotinoidic insecticides which have toxic effects on aphids due to their role as agonists on nicotinic acetylcholine receptors.

Alkaloids of the quinolizidine group are the main anti-nutritional substances in lupins, because of their bitter taste and toxicity (Michael, 2002, 2003; Sujak et al., 2006). Therefore, breeding for so called sweet lupins with a markedly reduced alkaloid content (<0.05% in seeds) was already initiated in the last century (Sengbusch, 1931; Fischer and Sengbusch, 1935; Sengbusch, 1942). With this improvement lupins turned to a food and fodder crop. Currently a threshold of 0.05% for feed and 0.02% for human nutrition are generally used (Reinhard et al., 2006; Jansen et al., 2009). However, the reduced alkaloid content in sweet lupins led to a high susceptibility to insect herbivores and plant pathogens, e.g. aphids (Wink, 1985, 1988; Wink and Witte, 1991). Aphids are important

pests in lupin cultivation causing severe yield losses up to 100% by feeding (Berlandier and Sweetingham, 2003; Kordan et al., 2008) and plant virus transmission (Thackray et al., 2004). Considering the parthenogenetic reproduction and viviparity, aphids have a high reproduction rate and short generation times (Blackman and Eastop, 2007) and a small initial infestation can rapidly lead to large populations resulting in significant plant damages (Webster et al., 2008). This will be additionally reinforced by the predicted climate change (Yamamura and Kiritani, 1998; Hullé et al., 2010).

Lupins can be infested by several aphid species (Holman, 2009). Especially the lupin aphid (*Macrosiphum albifrons*) can cause high infestation rates (Ferguson, 1994). The lupin aphid is feeding monophaguous on lupin species and is therefore well adapted to a high quinolizidine alkaloid level, which has toxic effects on non-adapted aphid species. Besides this *M. albifrons* sequesters the alkaloids and uses them for defense against predators (Wink and Roemer, 1986; Gruppe and Roemer, 1988; Emrich and Wink, 1992; Wink and Mohamed, 2003). In Australia, which is one of the main lupin growing areas, the polyphagous cowpea aphid (*Aphis craccivora*) is ubiquitous and causes severe yield losses on sweet genotypes (Zehnder et al., 2001). In addition, the green peach aphid (*Myzus persicae*) and the black bean aphid (*Aphis fabae*) as generalists and the pea aphid (*Acyrthosiphon pisum*), which is a specialist on different legume species, are able to infest and cause damages in sweet lupins, while they are unable to colonize alkaloid rich lupins (Gruppe and Roemer, 1988; Wink and Witte, 1991; Edwards, 2001; Kordan et al., 2008; Kordan et al., 2012). Aphid control based on insecticide application is expensive and harmful to the environment, which leads to the necessity for aphid resistant cultivars, especially in organic farming, where the use of insecticides is restricted. Therefore, breeding of narrow-leafed, aphid resistant lupins with a low alkaloid content is required.

Previous studies revealed genotypic differences in aphid susceptibility of narrow-leafed lupins, e.g. the sweet lupin Kalya was described as resistant (Zehnder et al., 2001; Berlandier and Sweetingham, 2003; Edwards et al., 2003; Adhikari et al., 2012). Therefore, the present study aimed at (i) identifying additional genotypes with different levels of susceptibility to *M. albifrons, M. persicae, A. fabae, A. craccivora* and *A. pisum* and (ii) to get information whether differences in aphid multiplication are related to the total alkaloid content or the alkaloid composition, respectively. To get information on this, the multiplication of the above mentioned aphid species on 46 genotypes of narrow-leafed lupins was investigated, and subsequently the alkaloid content and composition in the leaves was determined by gas chromatography/mass spectrometry (GC/MS).

2.3. Material & Methods

Plant material

A number of 46 *Lupinus angustifolius* L. genotypes was investigated, i.e. 22 cultivars (Azuro, Bora, Boregine, Borlu, Boruta, Coromup, Gunyidi, Haagena, Haags Blaue, Jenabillup, Kalya, Mandelup, Myallie, Paulsens Blaue, Probor, PSG Ostsaat Blaue, Quillinock, Rotblühende von Merkel, Sanabor, Sonate, Tallerack and Vitabor), 10 breeding lines (Bo073109/11, Bo083521AR, Bo103354/11, Bo103375, Bo103377, Bo113311, Bo113343, Bo113344, Bo113346 and Bo9027) and 14 gene bank accessions (L27254, L27479, LUP106/73, LUP141/80, LUP155/80, PI237721, PI255472, PI274814, PI274817, PI300023, PI308616, PI308619, PI383249 and PI384598). Plants were cultivated in growth chambers under controlled conditions at a temperature of 20°C under long-day conditions (16h light, intensity 10000 LUX, relative humidity of 60%) without any pesticide treatments. For pre-germination of the 46 narrow-leafed lupin genotypes, 20 seeds with 10 seeds per pot (11x11x11 cm) were cultivated in standard soil (Einheitserde Classic Profi Substrat CL T SM Sandfein; Einheitserde Werkverband, Sinntal, Germany). After 7 days, 10 seedlings were transferred in a single pot (11.3x11.3x21.5cm), each. The investigations were conducted at the developmental stage BBCH 30-35 (Dracup and Kirby, 1996).

Aphids

The multiplication rate on the above mentioned genotypes was estimated for the lupin aphid (*Macrosiphum albifrons*), the black bean aphid (*Aphis fabae*), the cowpea aphid (*Aphis craccivora*), the green peach aphid (*Myzus persicae*) and the pea aphid (*Acyrthosiphon pisum*). Aphid rearing was conducted in the greenhouse in plexiglas cages (50 x 60 x 65cm) under long-day conditions (16h light, sodium high pressure lamps) in climatized cabins with temperatures between 20° and 22° C and a relative humidity between 45% and 65%. *A. fabae*, *M. albifrons* and *M. persicae* were reared on *L. angustifolius* cv. Boregine, and *A. pisum* on *L. angustifolius* breeding strain Bo083521AR. Since the adaption and propagation of *A. craccivora* on *L. angustifolius* genotypes was not effective, rearing was conducted on faba bean (*Vicia faba*) cv. Scirocco.

Assessment of aphid multiplication

The experiments were conducted in growth chambers under controlled conditions at 16h light and 8h darkness at 20°C. The genotypes were separated in 2 sets with the breeding line Bo083521AR as a control for aphid multiplication, and the sets were investigated

consecutively. The experiments were repeated three times with 10 plants per genotype and aphid species combination, i.e. in total 30 plants per combination. Each replication was divided in 5 randomized blocks, separated by transparent plastic partition walls, and in each block each genotype was represented two times. Apterous female aphids were collected from the rearing by using a damped marten-hair brush (size 0). Four aphids of *A. fabae, A. craccivora, A. pisum* and *M. persicae* and, because of the higher multiplication rate, two aphids for *M. albifrons*, respectively, were set on each plant in each trial and the number of aphids was assessed after 7 and 14 days past infestation (dpi). Due to the differences in the number of aphids in the starting populations, aphid multiplication was defined as more than 2 aphids per day for *M. albifrons*, and more than 4 aphids per day for *A. fabae, A. pisum, A. craccivora* and *M. persicae*. For the statistical analysis of the population development the average ordinate (AO) of the population development curve was calculated displaying the number of aphids per day, according to the formula described by (Moll et al., 1996):

$$AO = \frac{1}{D} \times \sum_{i=1}^{t-1} \frac{1}{2} (N_i + N_{i+1}) \times d_i$$

where
D : number of days between first and last scoring date
i : scoring date (i = 1 … t)
N_i : number of aphids on scoring date i
d_i : number of days between the scoring days.

<u>Analysis of alkaloid content and composition</u>

After completion of the aphid assessment 14dpi, the leaves of all 10 plants per genotype were sampled and merged. In pre-tests (data not shown) the alkaloid content of infested and control plants of a subset of genotypes has been analyzed, and no significant differences were found. Therefore, the leaves of infested plants were used for the analysis of alkaloids. The leaves were frozen in liquid nitrogen and stored at -80°C for at least 72h, followed by lyophilization for approximately 24h. The samples were stored at room temperature and analyzed according to a modified method of (Torres et al., 2002). The lyophilized leaves were ground with the variable speed rotor mill ‘Pulverisette 14’ (Fritsch, Idar-Oberstein, Germany). A sample of 0,05g for genotypes with a high alkaloid level and 0.5g for sweet genotypes was blended with 10ml 1N HCl and stirred for 1h. As an internal standard for the alkaloid quantification 50µl caffeine solution (2mg ml^{-1} methyl alcohol;

Sigma-Aldrich, Steinheim, Germany) was added, and the homogenate was centrifuged for 20min at 10000 x g. Subsequently, the supernatant was adjusted to pH≥13 by adding 1ml of ammonia (25%) and 1.7ml 6N NaOH, and was applied to an extraction column

filled with Hydromatrix (high purity, inert diatomaceous earth sorbent, Agilent Technologies, Waldbronn, Germany). Alkaloids were eluted with methylene chloride. The solvent was removed by evaporation, the residue was solved in 200µl methyl alcohol and analyzed in the gas chromatograph '7890A GC' (Agilent Technologies) with flame ionization detector (FID). To separate the alkaloids, a capillary column DB-1 (25m x 0.20mm x 0.33µm, Agilent J&W) was used. For calibration and alkaloid quantification, pure lupanine, 13-hydroxylupanine (both provided by HU Jürgens) and sparteine (Sigma-Aldrich) were used, and caffeine as an internal standard. The quantification of all other alkaloids was conducted by using the calibration curve of lupanine. Alkaloid identification was performed by mass spectrometry (MSD 5975C; Agilent Technologies), comparing the mass spectra and Kovats retention indices with data of Wink et al. (1995).

Statistical analysis

Results of the alkaloid analysis were investigated for outliers based on the Jackknife distance (JD) method by using the multivariate methods option of JMP®Genomics 5.1 software (SAS Institute Inc., Cary, NC, USA). Values showing a JD>20 were excluded from the calculation. Because the alkaloid content data were not normally distributed and the number of samples was not equal for all genotypes, the procedure NPAR1WAY of SAS 9.4 (SAS Institute Inc.) was applied for nonparametric statistical analysis, using the DSCF option that requests the Dwass, Steel, Critchlow-Fligner multiple comparison procedure, which is based on pairwise two-sample rankings to find significant differences between genotypes.

To investigate the influence of the alkaloid content and composition for differences between the different aphid species a principal component analysis (PCA) was conducted by using the JMP®Genomics principal component analysis option. As the data of the aphid multiplication turned out to be not normally distributed, a nonparametric multiple comparison of the genotypes was conducted, using the Dunn test for all pairs for joint ranks of JMP®Genomics. For calculating Spearman's correlation coefficient the Proc CORR application of SAS was used, and the multiple regression analysis was conducted by using the Proc REG procedure. To obtain a linear relationship between the alkaloid content and aphid multiplication a log-normal transformation ln(x+1) of the data was done.

2.4. Results

In the first step to get information whether the alkaloid composition is influenced specifically by different aphids a principal component analysis (PCA) was conducted. The PCA (Figure 2.4-1) revealed no aphid specific differences with respect to the alkaloid composition. Therefore, the mean alkaloid content and composition was determined on all infested plants of a genotype irrespective of the aphid species (Table **2.4-1** and Table 2.4-2). The breeding line Bo083521AR showed the lowest (64.1 µg/g dry leaf matter) and L 27254 the highest (20205.3 µg/g dry leaf matter) total alkaloid content of the genotypes investigated. Generally, 13-hydroxylupanine had the largest share of the total alkaloid content, followed by angustifoline and 13-tigloyloxylupanine. Furthermore, the alkaloids lupanine, 13-trans-cinnamoyloxylupanine, 13-cis-cinnamoyloxylupanine, 13-benzoyloxylupanine, isolupanine and tetrahydrorhombifoline were detected in all genotypes investigated. Multiflorine (≤40 µg/g) and sparteine (≤24 µg/g) were only found in some genotypes and in small quantities. Other alkaloids, e.g. ammodendrine and 11,12-seco-12,13-didehydromultiflorine, were found in traces but below the detection limit (data not shown).

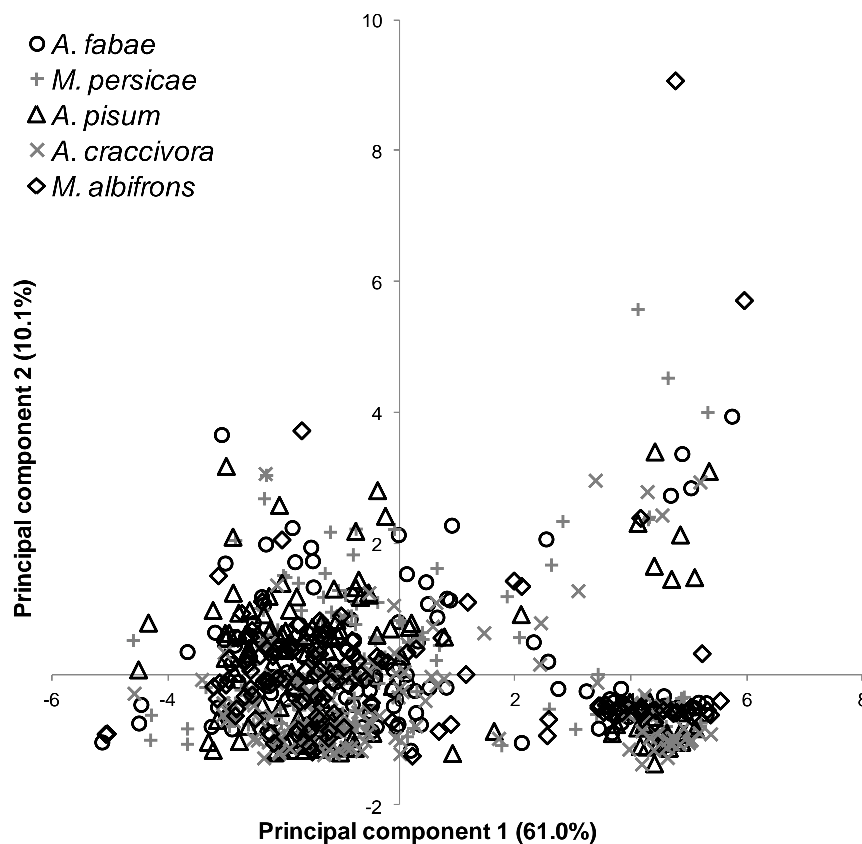

Figure 2.4-1: Principal component analysis (PCA) of the alkaloids in the leaves of 46 genotypes of *L. angustifolius* infested by *A. fabae, M. persicae, A. pisum, A. craccivora* and *M. albifrons*

Table 2.4-1: Total alkaloid content and content of different alkaloids (mean ± SE in µg/g dry matter) in leaves of 46 *L. angustifolius* genotypes (N = No. of samples*)

Genotype	N	Total Mean ± SE		13-hydroxylupanine Mean ± SE		13-tigloyloxylupanine Mean ± SE		13-benzoyloxylupanine Mean ± SE		13-cis-cinnamoyloxylupanine Mean ± SE		13-trans-cinnamoyloxylupanine Mean ± SE	
Bo083521AR	26	64.1 ± 8.5	e	19.9 ± 1.8	d	7.2 ± 1.3	e	3.2 ± 0.9	c	1.3 ± 0.7	c	5.6 ± 1.8	b
Tallerack	14	135.4 ± 16.5	de	42.0 ± 4.1	c	22.3 ± 2.7	d	11.1 ± 3.5	bc	4.7 ± 2.0	c	10.5 ± 4.0	b
Coromup	15	170.1 ± 17.8	de	97.3 ± 10.4	c	11.8 ± 1.3	de	2.9 ± 0.7	c	1.1 ± 0.4	c	5.8 ± 1.8	b
Gunyidi	15	180.9 ± 17.6	de	95.6 ± 8.6	c	17.4 ± 1.8	de	4.7 ± 1.2	c	2.7 ± 0.9	c	6.5 ± 2.2	b
Haagena	14	196.0 ± 21.1	de	71.5 ± 8.7	c	31.1 ± 4.8	d	18.6 ± 3.9	bc	5.2 ± 1.7	c	14.8 ± 3.9	b
Boregine	12	203.0 ± 10.6	d	74.2 ± 10.3	c	26.8 ± 3.4	d	20.9 ± 4.8	bc	1.3 ± 0.5	c	19.4 ± 4.8	b
Vitabor	12	225.4 ± 47.0	de	86.5 ± 19.2	c	24.9 ± 4.9	d	16.3 ± 3.4	bc	5.6 ± 1.9	c	28.9 ± 9.9	b
Bo113346	15	241.3 ± 26.4	d	128.1 ± 12.5	c	43.7 ± 5.1	d	5.4 ± 1.3	c	4.2 ± 1.1	c	5.8 ± 1.7	b
PI308616	12	246.1 ± 25.8	d	126.3 ± 16.7	c	23.1 ± 1.9	d	7.0 ± 1.2	bc	4.7 ± 0.9	c	10.2 ± 2.9	b
Mandelup	14	247.9 ± 16.7	d	132.0 ± 10.2	c	30.1 ± 3.0	d	4.0 ± 1.2	c	2.4 ± 0.7	c	6.0 ± 2.0	b
Sonate	15	254.0 ± 16.9	d	149.7 ± 12.7	c	45.5 ± 4.2	d	7.0 ± 1.6	c	1.1 ± 0.4	c	5.7 ± 1.2	b
PI274817	12	255.9 ± 11.9	d	58.2 ± 4.9	c	15.6 ± 2.0	d	6.9 ± 1.5	c	3.8 ± 0.7	c	11.4 ± 3.0	b
PI237721	12	257.3 ± 30.8	d	126.7 ± 16.2	c	30.7 ± 3.1	b	10.0 ± 1.7	bc	5.1 ± 1.0	c	13.9 ± 4.0	b
Bo113344	14	260.8 ± 17.2	d	134.2 ± 7.7	c	48.4 ± 5.5	c	7.1 ± 2.1	bc	4.8 ± 1.2	c	6.1 ± 1.6	b
Jenabillup	14	277.2 ± 34.5	d	148.9 ± 18.7	c	17.2 ± 2.7	de	6.2 ± 1.8	c	3.5 ± 0.9	c	11.1 ± 3.7	b
Myallie	15	297.7 ± 23.8	d	125.9 ± 9.5	c	56.4 ± 4.3	c	6.4 ± 1.1	c	5.3 ± 0.9	c	8.0 ± 1.7	b
Borlu	15	306.6 ± 41.1	d	111.9 ± 12.1	c	57.7 ± 12.5	cd	21.4 ± 5.5	bc	9.0 ± 3.1	c	22.0 ± 6.2	b
Bo113343	15	320.8 ± 22.5	de	168.7 ± 12.6	c	47.2 ± 3.6	cd	8.9 ± 2.1	bc	5.2 ± 1.1	c	11.4 ± 3.0	b
Probor	15	335.2 ± 45.3	de	143.0 ± 24.6	c	28.4 ± 4.6	d	20.5 ± 5.0	bc	8.3 ± 2.1	c	32.3 ± 9.3	b
Boruta	15	344.2 ± 50.2	de	177.5 ± 24.8	c	25.5 ± 3.7	d	7.7 ± 2.2	c	6.5 ± 1.5	c	21.4 ± 6.6	b
PI255472	14	350.6 ± 108.8	de	179.6 ± 57.1	c	31.5 ± 7.1	d	7.8 ± 1.8	c	6.6 ± 2.2	c	11.8 ± 3.4	b
Bora	15	417.1 ± 49.5	de	153.2 ± 16.6	c	75.5 ± 13.4	c	34.1 ± 6.8	bc	13.5 ± 3.9	c	38.2 ± 10.2	b
Kalya	15	430.5 ± 47.8	de	172.8 ± 23.6	c	61.3 ± 7.2	c	28.6 ± 4.8	bc	9.9 ± 2.7	c	42.1 ± 10.1	b
Sanabor	13	436.1 ± 17.3	de	233.4 ± 9.9	c	72.4 ± 3.6	c	8.4 ± 1.6	bc	5.8 ± 0.9	c	9.9 ± 2.2	b
Haags Blaue	15	457.6 ± 49.3	de	242.8 ± 25.6	c	54.3 ± 4.6	c	11.3 ± 3.2	bc	6.7 ± 1.4	c	17.8 ± 5.1	b
Quillinock	15	469.7 ± 60.4	de	265.0 ± 35.6	c	35.1 ± 2.9	d	9.2 ± 1.8	bc	6.4 ± 1.1	c	13.4 ± 3.2	b
Bo103377	15	483.1 ± 23.4	de	261.6 ± 14.4	c	67.9 ± 6.5	c	13.0 ± 3.0	bc	6.7 ± 1.3	c	12.8 ± 3.2	b
Bo103354/11	15	518.0 ± 57.0	de	266.3 ± 28.7	c	58.0 ± 5.3	c	9.3 ± 2.1	bc	7.4 ± 1.5	c	20.8 ± 6.4	b
Bo073109/11	15	534.1 ± 34.3	e	282.3 ± 15.3	c	57.0 ± 4.6	c	12.1 ± 3.2	bc	8.2 ± 1.7	c	21.2 ± 5.9	b
Bo113311	15	628.6 ± 41.7	de	324.6 ± 24.9	c	80.0 ± 4.8	c	15.7 ± 3.7	bc	7.6 ± 1.4	c	20.4 ± 5.8	b
Bo103375	15	707.8 ± 50.0	de	358.9 ± 26.7	c	87.5 ± 6.1	c	16.6 ± 3.7	bc	10.5 ± 2.1	c	27.2 ± 8.7	b
PI300023	13	755.6 ± 217.8	de	380.7 ± 105.9	bc	74.9 ± 20.0	c	12.0 ± 2.5	bc	23.0 ± 8.1	bc	32.6 ± 13.6	b
L27479	14	892.0 ± 41.3	d	472.4 ± 23.3	b	190.8 ± 14.6	b	19.8 ± 2.9	bc	18.9 ± 2.7	bc	25.9 ± 4.8	b

Different letters indicate significant differences between means in a column (P<0.05).

* Three replications per genotype/aphid combination = 15 samples. Outliers (Jackknife distance >20) were excluded from calculation.

Table 2.4-1 - continued: Total alkaloid content and content of different alkaloids (mean ± SE in µg/g dry matter) in leaves of 46 *L. angustifolius* genotypes (N = No. of samples*)

Genotype	N	Total Mean ± SE		13-hydroxylupanine Mean ± SE		13-tigloyloxylupanine Mean ± SE		13-benzoyloxylupanine Mean ± SE		13-cis-cinnamoyloxylupanine Mean ± SE		13-trans-cinnamoyloxylupanine Mean ± SE	
LUP141/80	14	1963.4 ± 308.3	cd	840.7 ± 143.8	b	176.6 ± 17.5	b	37.0 ± 5.6	b	72.7 ± 13.9	b	144.4 ± 28.3	ab
PI384598	15	3771.5 ± 570.7	c	1993.0 ± 300.0	b	223.0 ± 38.8	b	19.7 ± 4.2	bc	70.0 ± 13.1	b	71.6 ± 19.2	b
PI308619	14	9736.8 ± 687.9	b	3804.5 ± 337.9	ab	615.0 ± 65.3	a	185.3 ± 26.1	a	506.4 ± 116.5	a	1601.1 ± 444.8	a
Azuro	15	9864.7 ± 734.3	b	3761.0 ± 528.7	ab	605.4 ± 73.9	a	192.2 ± 29.4	a	488.6 ± 109.6	a	1570.0 ± 459.7	a
LUP155/80	14	12054.6 ± 923.7	b	3912.7 ± 413.9	ab	676.6 ± 71.1	a	228.8 ± 34.6	a	651.1 ± 139.0	a	3038.4 ± 824.9	a
Rotbl. v. Merkel	15	12118.9 ± 1132.9	ab	4309.9 ± 498.3	ab	883.2 ± 105.9	a	201.3 ± 36.4	a	654.3 ± 134.2	a	2101.9 ± 619.9	a
LUP106/73	15	12880.4 ± 889.6	ab	5520.1 ± 661.5	a	843.2 ± 90.5	a	251.1 ± 44.2	a	583.1 ± 122.6	a	2386.3 ± 683.0	a
Paulsens Blaue	15	12947.6 ± 820.0	ab	4878.6 ± 495.4	a	1028.5 ± 107.5	a	235.2 ± 38.6	a	761.4 ± 150.6	a	2027.0 ± 539.9	a
PI274814	14	13210.8 ± 841.0	ab	4859.2 ± 565.8	a	752.0 ± 88.8	a	233.1 ± 41.1	a	663.6 ± 128.2	a	2364.5 ± 717.7	a
PI383249	15	13327.6 ± 1047.2	ab	6722.1 ± 780.3	a	410.0 ± 46.8	b	160.8 ± 21.2	a	671.1 ± 133.1	a	2065.1 ± 661.1	a
PSG Ostsaat Bl.	15	13509.7 ± 997.0	ab	4435.1 ± 559.2	ab	869.0 ± 106.5	a	242.9 ± 36.7	a	708.3 ± 139.2	a	2102.2 ± 506.9	a
Bo9027	15	14051.9 ± 1098.1	ab	4967.6 ± 584.4	a	751.1 ± 78.8	a	221.9 ± 38.0	a	617.3 ± 142.7	a	2074.8 ± 610.5	a
L27254	15	20205.3 ± 1466.2	a	3432.8 ± 381.2	ab	665.2 ± 82.2	a	165.9 ± 21.0	a	394.2 ± 64.4	a	1153.2 ± 335.6	a

Different letters indicate significant differences between means in a column (P<0.05).

* Three replications per genotype/aphid combination = 15 samples. Outliers (Jackknife distance >20) were excluded from calculation.

Table 2.4-2: Content of different alkaloids (mean ± SE in µg/g dry matter) in leaves of 46 *L. angustifolius* genotypes

Genotype	N	Lupanine Mean ± SE		Angustifoline Mean ± SE		Isolupanine Mean ± SE		Tetrahydrorhombifoline Mean ± SE		Multiflorine Mean ± SE		Sparteine Mean ± SE		Lusitanine Mean ± SE	
Bo083521AR	26	10.2 ± 1.8	e	6.3 ± 1.2	e	6.3 ± 1.1	fg	3.0 ± 1.1	b	1.0 ± 0.6	b	0.2 ± 0.1	a	0.0 ± 0.0.	b
Tallerack	14	11.8 ± 2.2	e	11.5 ± 2.4	de	17.9 ± 2.1	ef	3.7 ± 1.6	b	0.0 ± 0.0	b	0.0 ± 0.0	a	0.0 ± 0.0	b
Coromup	15	11.3 ± 1.3	e	26.4 ± 4.7	d	10.6 ± 1.1	fg	2.2 ± 0.6	b	0.1 ± 0.1	b	0.6 ± 0.2	a	0.0 ± 0.0	b
Gunyidi	15	11.8 ± 2.2	e	27.8 ± 3.8	d	4.7 ± 0.5	g	1.9 ± 0.7	b	7.0 ± 2.4	b	0.8 ± 0.2	a	0.0 ± 0.0	b
Haagena	14	12.8 ± 1.7	e	21.2 ± 3.7	de	16.8 ± 1.9	ef	3.9 ± 1.6	b	0.0 ± 0.0	b	0.0 ± 0.0	a	0.0 ± 0.0	b
Boregine	12	13.9 ± 1.6	e	19.1 ± 2.6	de	22.1 ± 1.7	ef	5.4 ± 2.0	b	0.0 ± 0.0	b	0.0 ± 0.0	a	0.0 ± 0.0	b
Vitabor	12	13.4 ± 2.2	e	27.1 ± 8.4	de	17.0 ± 3.1	efg	4.7 ± 1.7	b	1.1 ± 1.1	b	0.0 ± 0.0	a	0.0 ± 0.0	b
Bo113346	15	11.0 ± 2.5	e	35.6 ± 5.6	cd	4.5 ± 0.5	g	2.2 ± 0.8	b	0.1 ± 0.1	b	0.7 ± 0.2	a	0.0 ± 0.0	b
PI308616	12	22.1 ± 2.7	de	36.2 ± 6.1	cd	15.4 ± 1.7	efg	0.7 ± 0.4	b	0.1 ± 0.1	b	0.5 ± 0.2	a	0.0 ± 0.0	b
Mandelup	14	16.0 ± 2.7	e	34.7 ± 4.6	cd	18.6 ± 1.1	ef	3.1 ± 1.4	b	0.6 ± 0.4	b	0.6 ± 0.2	a	0.0 ± 0.0	b
Sonate	15	7.2 ± 0.9	e	25.1 ± 2.3	d	9.9 ± 1.3	fg	1.6 ± 0.5	b	0.2 ± 0.1	b	0.9 ± 0.3	a	0.0 ± 0.0	b
PI274817	12	14.5 ± 1.1	e	14.9 ± 1.7	de	66.6 ± 1.9	d	1.3 ± 0.6	b	0.0 ± 0.0	b	1.7 ± 0.6	a	60.8 ± 11.6	a

Different letters indicate significant differences between means in a column (P<0.05).

* Three replications per genotype/aphid combination = 15 samples. Outliers (Jackknife distance >20) were excluded from calculation.

Table 2.4-2 - continued: Content of different alkaloids (mean ± SE in µg/g dry matter) in leaves of 46 *L. angustifolius* genotypes

Genotype	N	Lupanine Mean ± SE		Angustifoline Mean ± SE		Isolupanine Mean ± SE		Tetrahydrorhombifoline Mean ± SE		Multiflorine Mean ± SE		Sparteine Mean ± SE		Lusitanine Mean ± SE	
PI237721	12	12.9 ± 1.8	e	38.9 ± 5.9	cd	16.1 ± 1.9	efg	1.6 ± 0.6	b	0.3 ± 0.3	b	1.2 ± 0.3	a	0.0 ± 0.0	b
Bo113344	14	9.3 ± 1.7	e	34.5 ± 3.5	cd	13.5 ± 0.6	fg	2.1 ± 0.7	b	0.1 ± 0.1	b	0.8 ± 0.2	a	0.0 ± 0.0	b
Jenabillup	14	22.8 ± 2.6	de	47.5 ± 7.9	cd	16.1 ± 1.7	ef	2.4 ± 0.7	b	0.9 ± 0.2	b	0.7 ± 0.2	a	0.0 ± 0.0	b
Myallie	15	13.1 ± 2.2	e	32.4 ± 4.1	d	48.0 ± 9.9	de	1.5 ± 0.5	b	0.1 ± 0.1	b	0.7 ± 0.2	a	0.0 ± 0.0	b
Borlu	15	15.0 ± 2.1	e	25.3 ± 3.9	de	30.7 ± 6.4	ef	5.4 ± 1.8	b	8.2 ± 1.8	b	0.0 ± 0.0	a	0.0 ± 0.0	b
Bo113343	15	10.0 ± 1.1	e	48.6 ± 7.5	cd	16.6 ± 1.3	ef	2.4 ± 0.8	b	0.1 ± 0.1	b	1.8 ± 1.1	a	0.0 ± 0.0	b
Probor	15	25.4 ± 3.1	de	43.6 ± 8.7	cd	25.6 ± 3.4	ef	7.2 ± 1.8	b	0.9 ± 0.9	b	0.0 ± 0.0	a	0.0 ± 0.0	b
Boruta	15	21.5 ± 3.4	de	60.7 ± 10.6	cd	19.8 ± 2.5	ef	2.1 ± 0.7	b	0.8 ± 0.3	b	0.7 ± 0.2	a	0.0 ± 0.0	b
PI255472	14	35.2 ± 14.4	e	56.5 ± 23.0	de	19.1 ± 5.3	fg	1.7 ± 0.5	b	0.0 ± 0.0	b	0.9 ± 0.2	a	0.0 ± 0.0	b
Bora	15	17.9 ± 2.4	de	40.5 ± 5.5	cd	33.7 ± 3.2	e	6.3 ± 1.7	b	4.0 ± 1.3	b	0.2 ± 0.2	a	0.0 ± 0.0	b
Kalya	15	21.2 ± 2.3	de	54.5 ± 9.9	cd	32.9 ± 3.8	e	6.0 ± 1.6	b	1.4 ± 1.0	b	0.0 ± 0.0	a	0.0 ± 0.0	b
Sanabor	13	16.0 ± 1.6	e	64.5 ± 4.0	cd	22.2 ± 1.4	ef	2.3 ± 0.7	b	0.1 ± 0.1	b	1.0 ± 0.3	a	0.0 ± 0.0	b
Haags Blaue	15	20.6 ± 3.4	de	74.0 ± 12.3	cd	26.3 ± 2.8	ef	2.9 ± 0.8	b	0.2 ± 0.1	b	0.7 ± 0.2	a	0.0 ± 0.0	b
Quillinock	15	31.0 ± 4.6	de	75.8 ± 12.4	cd	29.0 ± 3.8	ef	2.9 ± 0.7	b	0.6 ± 0.2	b	1.3 ± 0.5	a	0.0 ± 0.0	b
Bo103377	15	30.8 ± 2.7	de	63.8 ± 6.6	cd	22.2 ± 2.2	ef	3.3 ± 0.9	b	0.2 ± 0.1	b	0.8 ± 0.2	a	0.0 ± 0.0	b
Bo103354/11	15	30.9 ± 4.2	de	81.9 ± 15.3	cd	37.0 ± 3.6	de	3.3 ± 1.0	b	0.9 ± 0.3	b	2.2 ± 0.5	a	0.0 ± 0.0	b
Bo073109/11	15	29.7 ± 3.2	de	84.6 ± 10.7	cd	34.2 ± 2.3	de	3.3 ± 0.7	b	0.3 ± 0.1	b	1.3 ± 0.3	a	0.0 ± 0.0	b
Bo113311	15	31.0 ± 2.6	de	104.1 ± 12.6	c	39.2 ± 3.0	de	4.0 ± 1.0	b	1.0 ± 0.5	b	1.1 ± 0.2	a	0.0 ± 0.0	b
Bo103375	15	35.3 ± 3.4	d	119.3 ± 12.5	bc	46.5 ± 4.1	de	4.2 ± 0.9	b	0.7 ± 0.2	b	1.0 ± 0.2	a	0.0 ± 0.0	b
PI300023	13	79.7 ± 29.1	cd	124.2 ± 42.2	bc	24.1 ± 4.9	ef	2.1 ± 0.7	b	1.5 ± 1.1	b	0.9 ± 0.3	a	0.0 ± 0.0	b
L27479	14	26.4 ± 1.7	de	91.9 ± 5.7	c	40.9 ± 2.0	de	3.5 ± 1.0	b	0.9 ± 0.3	b	0.8 ± 0.2	a	0.0 ± 0.0	b
LUP141/80	14	310.0 ± 80.5	c	327.6 ± 56.7	b	50.2 ± 8.6	de	3.6 ± 1.4	b	0.2 ± 0.2	b	0.7 ± 0.3	a	0.0 ± 0.0	b
PI384598	15	528.0 ± 88.3	bc	543.0 ± 93.2	ab	262.0 ± 86.9	bc	57.6 ± 30.4	b	1.0 ± 0.3	b	2.6 ± 0.6	a	0.0 ± 0.0	b
PI308619	14	1115.6 ± 105.8	b	1571.9 ± 151.0	ab	274.3 ± 13.9	c	62.8 ± 15.6	b	0.0 ± 0.0	b	0.0 ± 0.0	a	0.0 ± 0.0	b
Azuro	15	1256.5 ± 116.0	ab	1642.5 ± 176.2	a	280.5 ± 16.4	c	66.8 ± 16.0	b	0.0 ± 0.0	b	1.3 ± 1.3	a	0.0 ± 0.0	b
LUP155/80	14	1525.4 ± 106.6	ab	1690.6 ± 157.5	ab	277.5 ± 13.4	c	52.8 ± 15.0	b	0.0 ± 0.0	b	0.7 ± 0.7	a	0.0 ± 0.0	b
Rotbl. v. Merkel	15	1754.7 ± 186.2	ab	1861.6 ± 236.2	a	279.1 ± 16.5	c	72.3 ± 16.1	b	0.0 ± 0.0	b	0.6 ± 0.6	a	0.0 ± 0.0	b
LUP106/73	15	1478.0 ± 154.9	ab	1630.1 ± 141.4	a	108.5 ± 14.3	d	62.2 ± 15.5	b	9.0 ± 9.0	b	8.9 ± 7.9	a	0.0 ± 0.0	b
Paulsens Blaue	15	1654.1 ± 147.3	ab	1988.6 ± 198.2	a	296.2 ± 19.5	c	77.0 ± 17.9	b	0.0 ± 0.0	b	1.2 ± 1.2	a	0.0 ± 0.0	b
PI274814	14	2078.5 ± 157.3	ab	1860.1 ± 195.0	a	328.0 ± 20.0	bc	71.7 ± 19.5	b	0.0 ± 0.0	b	0.0 ± 0.0	a	0.0 ± 0.0	b
PI383249	15	1193.1 ± 144.9	bc	1904.0 ± 173.0	a	122.0 ± 16.0	d	78.6 ± 18.4	b	0.0 ± 0.0	b	0.8 ± 0.8	a	0.0 ± 0.0	b
PSG Ostsaat Bl.	15	2623.2 ± 213.2	a	1911.0 ± 203.3	a	542.4 ± 37.8	b	75.6 ± 18.2	b	0.0 ± 0.0	b	0.0 ± 0.0	a	0.0 ± 0.0	b
Bo9027	15	2316.4 ± 227.8	ab	2503.8 ± 253.0	a	523.9 ± 31.8	bc	75.0 ± 14.3	b	0.0 ± 0.0	b	0.0 ± 0.0	a	0.0 ± 0.0	b
L27254	15	2247.6 ± 190.6	ab	257.1 ± 17.3	b	9477.8 ± 802.8	a	2347.6 ± 485.8	a	40.0 ± 13.5	a	24.0 ± 11.1	a	0.0 ± 0.0	b

Different letters indicate significant differences between means in a column ($P<0.05$).

* Three replications per genotype/aphid combination = 15 samples. Outliers (Jackknife distance >20) were excluded from calculation.

Genotype specific differences in the alkaloid composition were observed. Lusitanine was only detected in the genotype PI 274817 with an amount of 60.8 µg/g dry leaf matter. For L 27254, the genotype with the highest total alkaloid content, a remarkably high amount of isolupanine and tetrahydrorhombifoline was detected, and also the highest content of multiflorine and sparteine, while these were identified for most of the genotypes investigated in traces, only. Coincidentally the amount of angustifoline was markedly reduced and also a lower content of 13-cis-cinnamoyloxylupanine and 13-trans-cinnamoyloxylupanine was detected. In the leaves of PI383249 the highest amount of 13-hydroxylupanine was found, while the content of 13-tigloyloxylupanine was lower compared to genotypes with a similar total alkaloid content.

The multiplication of the different aphid species (Table 2.4-3) showed significant genotype specific differences. *M. albifrons* showed a multiplication on all genotypes investigated with quantitative variation in the infestation level but no resistant genotype was observed. In some cases, especially on genotype PI384598, the multiplication of *M. albifrons* damaged the plants severely and led to their dieback, followed by the emigration of the aphids to the adjacent plants. *A. fabae, M. persicae, A. pisum* and *A. craccivora* showed gradual differences between the genotypes investigated, and in a number of genotypes no multiplication was observed. In general, the polyphagous aphid species *A. fabae* and *M. persicae* were able to infest a higher number of genotypes compared with *A. pisum* and *A. craccivora,* and also generated a higher number of progenies. The highest number of individuals of *A. fabae, M. persicae, A. pisum* and *A. craccivora* was observed on the breeding line Bo083521AR, and the highest total multiplication rate per day on this genotype was found for *A. fabae*. On the sweet genotypes Boregine, Bora, Borlu and Kalya no or only a very limited aphid multiplication was observed.

To estimate the relations between aphid multiplication and the content of different alkaloids, Spearman's correlation coefficient was calculated for the AO (aphids per day) of the different aphid species. This was conducted on 33 genotypes containing a reduced alkaloid level because genotypes with an alkaloid content higher than 1000 µg/g dry leaf matter suppressed the multiplication of *A. fabae, M. persicae, A. craccivora* and *A. pisum* completely (Table 2.4-3). Significant negative correlations were observed for *A. fabae, M. persicae, A. pisum* and *A. craccivora* for the total amount of alkaloids as well as for 13-hydroxylupanine and its esters, 13-tigloyloxylupanine, 13-benzoyloxylupanine, 13-cis-cinnamoyloxylupanine and 13-trans-cinnamoyloxylupanine (Table 2.4-4). Significant negative correlations were also observed for lupanine, isolupanine and angustifoline. Only for *A. craccivora* no correlation with the multiplication and the content of 13-

benzoyloxylupanine, 13-cis-cinnamoyloxylupanine, 13-trans-cinnamoyloxylupanine and lupanine was detected and *M. persicae* showed no significant correlation with angustifoline. For sparteine a low but significant positive correlation was detected for *A. fabae*, *A. pisum* and *A. craccivora*. Tetrahydrorhombifoline and multiflorine, which were found only in traces in a few leave samples, showed no significant correlation with the multiplication of *M. persicae*, *A. pisum* and *A. craccivora*, only for *A. fabae* a negative correlation for Tetrahydrorhombifoline was calculated. In case of the average ordinate of the lupin aphid *M. albifrons* no correlation with the total alkaloid content was observed. In contrast to the other aphid species, a low but significantly positive correlation was calculated for 13-trans-cinnamoyloxylupanine. For 13-tigloyloxylupanine and sparteine a low but significant negative correlation was detected. It is remarkable, that despite the high significant correlation between the alkaloid content and the aphid multiplication some genotypes, in particular the cultivars Kalya, Bora and Borlu, showed a low multiplication and simultaneously a low alkaloid content.

As shown in Table 2.4-4 the aphid multiplication is also influenced by the alkaloid composition. Hence, a multiple linear regression was calculated (Table 2.4-5) to identify the major factors influencing the aphid multiplication. It was shown that for *A. fabae, M. persicae, A. craccivora* and *A. pisum* more than 60% of the variance of aphid multiplication is due to the alkaloids content and their composition, displayed by R-square values between 0.652 and 0.707. Especially 13-hydroxylupanine, its esters 13-tigloyloxylupanine, 13-benzoyloxylupanine, 13-cis-cinnamoyloxylupanine and 13-trans-cinnamoyloxylupanine have an impact on the aphid survival and population development, with aphid specific differences. For *M. albifrons* the regression of the whole model is significant but only a low part of the variance (R^2=0.315) is explained by the alkaloid composition. In the used regression model only sparteine has a significant influence in the multiplication of *M. albifrons*.

For breeding new varieties with reduced susceptibility against different aphid species biomarkers as the alkaloid content and the composition may be useful tools. Therefore, multiple regression models were calculated for all aphid species except *M. albifrons,* for which no genotype with reduced susceptibility was observed (Table 2.4-6). With the complete regression model a R^2-value of 0.447 was realized, and 13-tigloyloxylupanine showed the highest significant influence in the regression for the aphid multiplication (R^2 = 0.310).

Table 2.4-3: Multiplication (Number of plants (N)*, mean ± standard error (SE) of average ordinate (AO; aphids/day) of *A. pisum, A. craccivora, A. fabae, M. persicae* and *M. albifrons* on 46 *L. angustifolius* genotypes, sorted by total alkaloid content.

	A. fabae			*M. persicae*			*A. pisum*			*A. craccivora*			*M. albifrons*		
Genotype	N	Mean ± SE		N	Mean ± SE		N	Mean ± SE		N	Mean ± SE		N	Mean ± SE	
Bo083521AR	60	**127.8 ± 11.8**	a	60	**74.3 ± 9.2**	a	59	**76.3 ± 7.1**	a	59	**34.3 ± 6.2**	a	54	**76.6 ± 6.8**	ab
Tallerack	27	**15.1 ± 2.9**	bcdef	26	**9.8 ± 2.9**	cdefghi	26	1.1 ± 0.4	defghi	27	1.4 ± 0.3	cdefg	30	**101.2 ± 9.0**	a
Coromup	27	**33.1 ± 5.8**	abcd	30	**51.0 ± 6.6**	ab	30	**49.0 ± 6.0**	ab	30	**10.3 ± 1.7**	ab	30	**69.3 ± 9.2**	ab
Gunyidi	30	**53.5 ± 10.3**	abc	26	**23.6 ± 3.7**	abcde	29	**23.1 ± 6.5**	abc	30	**6.7 ± 1.5**	abc	30	**55.9 ± 7.3**	ab
Haagena	28	**5.4 ± 1.1**	efghi	25	2.3 ± 0.9	ghijklm	27	0.2 ± 0.1	ghi	28	1.8 ± 0.4	cdefg	30	**75.8 ± 9.9**	ab
Boregine	29	**4.7 ± 1.1**	efghi	26	1.0 ± 0.3	hijklm	27	0.4 ± 0.2	fghi	29	1.4 ± 0.5	cdefg	30	**105.9 ± 12.0**	a
Vitabor	30	**8.0 ± 1.5**	cdefgh	26	**9.6 ± 2.6**	cdefghi	26	1.6 ± 0.6	defghi	27	**6.6 ± 1.5**	abcd	30	**77.8 ± 10.9**	ab
Bo113346	30	**15.3 ± 2.1**	bcdef	27	**4.4 ± 0.9**	cdefghi	26	1.1 ± 0.2	defghi	29	1.5 ± 0.5	cdefg	28	**48.5 ± 5.5**	ab
PI308616	29	**15.9 ± 2.7**	bcdef	29	**16.3 ± 2.4**	abcdef	30	**9.7 ± 2.2**	abcd	30	3.7 ± 1.3	bcde	30	**59.4 ± 8.3**	ab
Mandelup	30	**31.8 ± 4.8**	abcd	30	**12.6 ± 2.5**	abcdefg	30	**4.7 ± 1.4**	cdefg	30	1.5 ± 0.5	cdefg	28	**85.1 ± 12.0**	ab
Sonate	29	**10.4 ± 2.0**	cdefg	26	2.7 ± 0.5	fghijkl	29	0.6 ± 0.2	efghi	30	0.4 ± 0.1	efg	30	**65.3 ± 9.5**	ab
PI274817	30	**77.0 ± 8.0**	ab	30	**25.9 ± 2.8**	abc	30	**27.0 ± 5.2**	ab	30	2.9 ± 0.6	bcdef	30	**63.9 ± 6.6**	ab
PI237721	28	**18.2 ± 2.3**	abcde	27	**14.5 ± 3.5**	abcdef	28	1.2 ± 0.3	defghi	30	0.9 ± 0.2	cdefg	30	**59.9 ± 6.0**	ab
Bo113344	28	**17.3 ± 3.2**	abcdef	30	**6.2 ± 1.2**	cdefghi	28	0.9 ± 0.2	defghi	30	0.5 ± 0.1	efg	22	**50.0 ± 6.1**	ab
Jenabillup	30	**27.1 ± 3.9**	abcd	26	**24.6 ± 2.8**	abcd	30	**14.7 ± 3.4**	abc	28	3.7 ± 0.7	abcd	28	**74.4 ± 8.2**	ab
Myallie	28	**23.2 ± 4.5**	abcde	29	**11.4 ± 2.9**	abcdefg	28	1.4 ± 0.5	defghi	28	0.6 ± 0.2	efg	30	**67.4 ± 9.5**	ab
Borlu	29	2.5 ± 0.4	fghi	28	0.7 ± 0.2	ijklm	30	0.1 ± 0.0	ghi	30	1.1 ± 0.3	cdefg	30	**66.4 ± 8.6**	ab
Bo113343	30	**14.4 ± 3.2**	bcdef	30	**4.9 ± 0.9**	cdefghi	29	1.2 ± 0.4	defghi	30	1.4 ± 0.4	cdefg	27	**50.1 ± 7.5**	ab
Probor	27	**6.7 ± 1.5**	efghi	23	**8.6 ± 2.4**	cdefghi	28	0.4 ± 0.2	fghi	27	2.3 ± 0.6	bcdef	30	**73.6 ± 9.2**	ab
Boruta	30	**23.2 ± 4.1**	abcde	29	**9.8 ± 2.1**	bcdefgh	27	**6.2 ± 1.9**	abcde	28	2.3 ± 0.6	cdefg	28	**54.7 ± 5.0**	ab
PI255472	28	**43.2 ± 7.5**	abcd	30	**11.1 ± 1.9**	abcdefg	28	**5.0 ± 1.0**	bcdef	30	2.2 ± 0.7	cdefg	29	**59.3 ± 7.9**	ab
Bora	29	0.9 ± 0.3	hi	26	1.8 ± 0.6	hijklm	30	0.2 ± 0.1	ghi	30	1.0 ± 0.2	cdefg	30	**76.2 ± 6.7**	ab
Kalya	28	1.1 ± 0.4	hi	26	0.6 ± 0.2	ijklm	30	0.3 ± 0.2	fghi	30	1.1 ± 0.3	cdefg	30	**87.4 ± 10.7**	ab
Sanabor	30	**8.8 ± 1.4**	cdefgh	29	2.7 ± 0.5	defghij	29	0.4 ± 0.1	efghi	30	0.3 ± 0.1	efg	29	**70.5 ± 8.0**	ab
Haags Blaue	29	**10.5 ± 1.7**	bcdef	29	3.8 ± 0.7	defghi	27	0.4 ± 0.1	efghi	30	0.7 ± 0.2	defg	30	**75.7 ± 11.7**	ab
Quillinock	30	**13.0 ± 2.1**	bcdef	30	**4.6 ± 0.8**	cdefghi	27	1.2 ± 0.4	defghi	28	0.8 ± 0.3	defg	29	**72.2 ± 10.0**	ab
Bo103377	29	3.7 ± 0.8	efghi	29	3.2 ± 0.5	defghi	26	0.9 ± 0.2	defghi	29	0.7 ± 0.2	defg	30	**87.6 ± 9.6**	a
Bo103354/11	30	**7.1 ± 1.6**	defgh	28	2.7 ± 0.5	efghijk	30	0.9 ± 0.3	defghi	30	0.9 ± 0.2	cdefg	23	**74.4 ± 12.4**	ab
Bo073109/11	30	**15.1 ± 3.4**	bcdef	29	**4.3 ± 0.9**	defghi	27	0.7 ± 0.1	defghi	30	1.7 ± 0.7	cdefg	29	**61.4 ± 9.5**	ab
Bo113311	29	3.1 ± 0.6	efghi	27	0.7 ± 0.2	ijklm	29	0.9 ± 0.2	defghi	30	0.9 ± 0.2	cdefg	30	**51.7 ± 7.9**	ab
Bo103375	29	3.0 ± 0.6	efghi	29	1.2 ± 0.3	hijklm	30	1.0 ± 0.3	defghi	30	1.2 ± 0.3	cdefg	30	**74.9 ± 7.3**	ab
PI300023	27	**17.0 ± 3.1**	bcdef	28	**10.3 ± 2.0**	abcdefgh	30	**4.1 ± 1.3**	cdefgh	29	1.7 ± 0.6	cdefg	30	**87.0 ± 9.7**	ab

Different letters indicate significant differences between means in a column (Dunn pairwise test for joint ranks, $P < 0.05$);

Genotypes with aphid multiplication (AO higher 4 for *A. fabae, M. persicae, A. pisum* and *A. craccivora*, and higher 2 for *M. albifrons*) are printed in bold.

* Three replications with 10 plants/genotype=30 plants/genotype. Plants dying back during assessment period were excluded from calculation.

Table 2.4-3 – continued: Multiplication (Number of plants (N)*, mean ± standard error (SE) of average ordinate (AO; aphids/day) of *A. pisum*, *A. craccivora*, *A. fabae*, *M. persicae* and *M. albifrons* on 46 *L. angustifolius* genotypes, sorted by total alkaloid content.

	A. fabae			*M. persicae*			*A. pisum*			*A. craccivora*			*M. albifrons*		
Genotype	N	Mean ± SE		N	Mean ± SE		N	Mean ± SE		N	Mean ± SE		N	Mean ± SE	
L27479	30	1.2 ± 0.3	ghi	29	0.8 ± 0.1	hijklm	29	0.4 ± 0.2	fghi	30	0.6 ± 0.2	efg	29	**51.1 ± 5.3**	ab
LUP141/80	30	3.0 ± 0.6	fghi	30	0.8 ± 0.2	hijklm	29	0.3 ± 0.1	ghi	30	0.4 ± 0.2	efg	28	**85.6 ± 11.0**	ab
PI384598	29	**7.7 ± 1.5**	defgh	27	1.1 ± 0.3	hijklm	27	0.5 ± 0.2	efghi	30	0.9 ± 0.3	defg	24	**38.3 ± 7.3**	b
PI308619	30	0.0 ± 0.0	i	29	0.1 ± 0.0	lm	30	0.1 ± 0.0	hi	29	0.1 ± 0.1	g	29	**81.4 ± 8.0**	ab
Azuro	29	0.0 ± 0.0	i	30	0.1 ± 0.0	lm	29	0.1 ± 0.1	ghi	30	0.1 ± 0.1	g	30	**93.3 ± 8.8**	a
LUP155/80	30	0.0 ± 0.0	i	30	0.1 ± 0.0	klm	30	0.0 ± 0.0	i	29	0.1 ± 0.1	fg	29	**85.3 ± 10.1**	ab
Rotbl. v. Merkel	29	0.0 ± 0.0	i	29	0.1 ± 0.0	lm	29	0.0 ± 0.0	hi	29	0.2 ± 0.1	efg	30	**81.1 ± 9.3**	ab
LUP106/73	30	0.1 ± 0.0	i	30	0.2 ± 0.1	klm	30	0.1 ± 0.0	ghi	30	0.2 ± 0.1	fg	30	**96.5 ± 10.8**	a
Paulsens Blaue	29	0.1 ± 0.0	i	30	0.0 ± 0.0	m	30	0.1 ± 0.1	ghi	29	0.1 ± 0.1	g	30	**99.2 ± 11.2**	a
PI274814	30	0.1 ± 0.0	i	30	0.2 ± 0.1	jklm	30	0.0 ± 0.0	i	29	0.2 ± 0.1	fg	30	**94.1 ± 7.8**	a
PI383249	30	0.6 ± 0.6	i	28	0.1 ± 0.1	lm	30	0.1 ± 0.0	ghi	30	0.1 ± 0.1	g	30	**66.7 ± 6.1**	ab
PSG Ostsaat Bl.	29	0.1 ± 0.0	i	30	0.1 ± 0.0	klm	29	0.1 ± 0.0	ghi	30	0.2 ± 0.1	fg	30	**108.0 ± 14.7**	a
Bo9027	30	0.5 ± 0.4	i	30	0.2 ± 0.1	klm	30	0.1 ± 0.0	ghi	30	0.2 ± 0.1	fg	30	**84.9 ± 9.6**	ab
L27254	30	0.1 ± 0.1	i	28	0.1 ± 0.0	klm	30	0.1 ± 0.1	ghi	29	0.1 ± 0.1	g	29	**71.5 ± 6.5**	ab

Different letters indicate significant differences between means in a column (Dunn pairwise test for joint ranks, $P < 0.05$);

Genotypes with aphid multiplication (AO higher 4 for *A. fabae, M. persicae, A. pisum* and *A. craccivora*, and higher 2 for *M. albifrons*) are printed in bold.

* Three replications with 10 plants/genotype=30 plants/genotype. Plants dying back during the assessment period were excluded from calculation.

Table 2.4-4: Correlation (Spearman's correlation coefficient (r) and p-value) of the log normal-transformed alkaloid content [ln(x+1)] in leaves of 33 *Lupinus angustifolius* L. genotypes with a mean total alkaloid content <1000 µg/g in dry leaf matter and the log normal-transformed Average Ordinate [ln(x+1)] of *A. fabae, M. persicae, A. pisum, A. craccivora* and *M. albifrons*

		A. fabae	*M. persicae*	*A. pisum*	*A. craccivora*	*M. albifrons*
Total	r	**-0.493**	**-0.350**	**-0.477**	**-0.422**	-0.078
	p-value	<.0001	0.0006	<.0001	<.0001	0.4584
13-hydroxylupanine	r	**-0.473**	**-0.425**	**-0.238**	**-0.526**	-0.100
	p-value	<.0001	<.0001	0.0223	<.0001	0.3406
13-tigloyloxylupanine	r	**-0.638**	**-0.510**	**-0.673**	**-0.398**	**-0.221**
	p-value	<.0001	<.0001	<.0001	<.0001	0.0331
13-benzoyloxylupanine	r	0.156	**0.294**	**0.289**	0.096	-0.032
	p-value	0.1409	0.0045	0.0052	0.3633	0.7632
13-cis-cinnamoyloxylupanine	r	**-0.375**	**-0.360**	**-0.633**	-0.077	0.164
	p-value	0.0003	0.0004	<.0001	0.4682	0.1159
13-trans-cinnamoyloxylupanine	r	**-0.293**	**-0.246**	**-0.563**	0.070	**0.282**
	p-value	0.0048	0.0183	<.0001	0.5122	0.0062
Lupanine	r	**-0.285**	**-0.276**	**-0.245**	-0.153	0.066
	p-value	0.0062	0.0077	0.0187	0.1484	0.5318
Isolupanine	r	**-0.382**	**-0.222**	**-0.361**	**-0.355**	-0.110
	p-value	0.0002	0.0335	0.0004	0.0006	0.2942
Angustifoline	r	**-0.295**	-0.194	**-0.365**	**-0.433**	-0.058
	p-value	0.0045	0.0644	0.0003	<.0001	0.5791
Tetrahydrorhombifoline	r	**-0.421**	-0.201	0.178	-0.094	-0.153
	p-value	<.0001	0.0545	0.0893	0.3762	0.1441
Multiflorine	r	-0.047	0.034	-0.045	0.043	0.024
	p-value	0.6553	0.7479	0.6721	0.685	0.821
Sparteine	r	**0.256**	0.106	**0.284**	**0.324**	**-0.387**
	p-value	0.0144	0.3161	0.0061	0.0017	0.0001
Lusitanine	r	**0.288**	**0.225**	0.202	0.084	0.004
	p-value	0.0056	0.0311	0.0532	0.4291	0.9667

Significant values ($P < 0.05$) between alkaloid and AO are printed in bold.

Table 2.4-5: Multiple linear regression analysis of the log normal-transformed Average Ordinate [ln(x+1)] of *A. fabae, M. persicae, A. pisum, A. craccivora* and *M. albifrons* and the log normal-transformed alkaloid content [ln(x+1)] in leaves of 33 *Lupinus angustifolius* L. genotypes with a total alkaloid content <1000 µg/g in the dry matter.

Variable	*A. fabae*			*M. persicae*			*A. pisum*			*A. craccivora*			*M. albifrons*		
	Estimate	SE	p-value	Estimate	SE	p-value	Estimate	SE	p-value	Estimate	SE	p-value	Estimate	SE	p-value
Intercept	**5.53**	**0.91**	**<.0001**	**6.67**	**0.94**	**<.0001**	**3.30**	**0.92**	**0.0006**	**2.30**	**0.60**	**0.0002**	**3.96**	**0.66**	**<.0001**
ln 13-hydroxylupanine	-0.48	0.30	0.1146	**-1.34**	**0.25**	**<.0001**	0.28	0.28	0.3209	-0.11	0.16	0.5053	0.16	0.21	0.4358
ln 13-tigloyloxylupanine	**-1.00**	**0.23**	**<.0001**	**-0.84**	**0.20**	**<.0001**	**-0.76**	**0.15**	**<.0001**	**-0.46**	**0.14**	**0.0022**	-0.08	0.09	0.4191
ln 13-benzoyloxylupanine	**0.09**	**0.03**	**0.0007**	0.03	0.03	0.3315	0.05	0.04	0.1435	**0.07**	**0.02**	**0.0025**	0.01	0.02	0.7259
ln 13-cis-cinnamoyloxylupanine	0.17	0.20	0.4042	-0.27	0.15	0.0841	-0.04	0.20	0.8491	**0.22**	**0.11**	**0.0489**	0.08	0.13	0.5162
ln 13-trans-cinnamoyloxylupanine	0.14	0.11	0.2201	-0.05	0.09	0.5841	-0.15	0.13	0.2405	**0.22**	**0.10**	**0.0282**	0.18	0.09	0.0581
ln lupanine	-0.05	0.21	0.8242	-0.01	0.18	0.9578	-0.09	0.23	0.7111	0.12	0.12	0.3254	0.05	0.18	0.7702
ln angustifoline	**0.46**	**0.13**	**0.0005**	**1.43**	**0.19**	**<.0001**	-0.31	0.30	0.3113	-0.23	0.20	0.2534	-0.22	0.12	0.0862
ln isolupanine	-0.07	0.19	0.7255	0.02	0.16	0.9234	0.02	0.18	0.9342	**-0.23**	**0.11**	**0.0492**	-0.07	0.14	0.6178
ln multiflorine	0.03	0.10	0.7989	0.19	0.11	0.0810	0.18	0.12	0.1371	0.10	0.11	0.3888	-0.11	0.15	0.4741
ln sparteine	0.33	0.20	0.1006	-0.22	0.22	0.3244	0.08	0.19	0.6590	**0.39**	**0.15**	**0.0128**	**-0.48**	**0.18**	**0.0079**
ln tetrahydrorhombifoline	**-0.45**	**0.13**	**0.0009**	**-0.55**	**0.14**	**0.0002**	-0.04	0.14	0.7621	0.05	0.09	0.6089	-0.03	0.08	0.6946
ln lusitanine	0.21	0.12	0.0820	0.08	0.12	0.4842	0.26	0.18	0.1386	0.05	0.11	0.6712	0.07	0.13	0.5675
F-statistic	15.70			13.85			12.33			14.20			3.06		
p-value	<.0001			<.0001			<.0001			<.0001			0.0014		
R^2	0.707			0.678			0.652			0.686			0.315		

Significant values (p<0.05) are printed in bold.

Table 2.4-6: Performance values for the best multiple linear regression model of each size class of the log normal-transformed Average Ordinate [ln(x+1)] of *A. fabae, M. persicae, A. pisum and A. craccivora* and the log normal-transformed alkaloid content [ln(x+1)] in leaves of 33 *Lupinus angustifolius* L. genotypes with a total alkaloid content <1000µg/g in the dry matter.

No. of variables in model	ln 13-tigloyloxylupanine	ln 13-benzoyloxylupanine	ln spartein	ln angustifolin	ln tetrahydrorhombifoline	ln lusitanine	ln multiflorin	ln 13-cis-cinnamoyloxylupanine	ln isolupanin	ln 13-hydroxylupanine	ln 13-trans-cinnamoyloxylupanine	ln lupanin	R2	F-statistic	p-value
1	x												0.3102	163.72	<.0001
2	x	x											0.3427	94.65	<.0001
3	x	x	x										0.3738	72.05	<.0001
4	x	x		x	x								0.4072	62.00	<.0001
5	x	x		x	x	x							0.4269	53.64	<.0001
6	x	x	x	x	x	x							0.4351	46.09	<.0001
7	x	x	x	x	x	x	x						0.4412	40.39	<.0001
8	x	x	x	x	x	x	x	x					0.4430	35.49	<.0001
9	x	x	x	x	x	x	x	x	x				0.4444	31.64	<.0001
10	x	x	x	x	x	x	x		x	x	x		0.4469	28.68	<.0001
11	x	x	x	x	x	x	x	x	x	x	x		0.4471	26.03	<.0001
12	x	x	x	x	x	x	x	x	x	x	x	x	0.4472	23.80	<.0001

2.5. Discussion

The lupin quinolizidine alkaloids have been investigated in detail already and their protective and inhibitory effect against herbivores is well described, especially against insects but also bacterial and fungal pathogens (Wink, 1987a, 1988, 1992; Wink et al., 1995). Quinolizidine alkaloids act as an agonist of muscarinic (mAChR; e.g. angustifoline) or nicotinic acetylcholine receptors (nAChR; e.g. lupanine) (Wink, 2000). Binding on nAChRs is also described for nicotine and neonicotinoids, e.g. Imidacloprid, the most important neonicotinoid insecticide with good systemic activity causing the insect to reduce or stop feeding and mobility (Sadeghi et al., 2009).

Differences in susceptibility for aphid damage are known for Australian lupin cultivars (French, 2004). Berlandier and Sweetingham (2003) showed in field trials in Western Australia that the cowpea aphid (*Aphis craccivora*), the green peach aphid (*Myzus persicae*) and the bluegreen aphid (*Acyrthosiphon kondoi*) are causing varying yield losses depending on the cultivar investigated. These findings are underlining the

results of the present study, where with exception of the specialized species *M. albifrons* significant differences in aphid infestation between the genotypes were observed (Table 2.4-3). Findings of Ferguson (1994) revealed, that an infestation with *M. albifrons* is only to a small extend influenced by biochemical differences between *Lupinus albus* cultivars. These results coincide with the current study, in which no *L. angustifolius* genotypes resistant to *M. albifrons* were detected. It was recorded by Wink and Roemer (1986) that alkaloids serve *M. albifrons* as a cue to find suitable host plants, and plants with a low alkaloid content are assumed to be less attractive. Results of the present study showed no reduced infestation on genotypes with a low alkaloid content, indicating that a low alkaloid content does not reduce the aphid multiplication. Results of Wink and Roemer (1986) and Gruppe and Roemer (1988) showed, that lupins containing the bicyclic quinolizidine alkaloid lupinine, e.g. *L. luteus*, are avoided by *M. albifrons*. This is an indication for the varying influence of different alkaloids on host plant acceptance of aphids and gives hint that not only the alkaloid content but its composition plays a major role in aphid infestation.

For all the aphids except *M. albifrons* the breeding line Bo083521AR with the lowest alkaloid content was most susceptible. A higher alkaloid content reduces the possibility for the aphids to multiply and it turned out that at more than 4000 µg alkaloid/g leaf dry matter no aphid multiplication is possible. This is indicating that the current breeding for low alkaloid content is increasing the problem of aphid susceptibility.

The results showed additionally, that *A. fabae* was able to infest most of the *L. angustifolius* genotypes with a low alkaloid content, followed by *M. persicae. Acyrthosiphon pisum* and *A. craccivora* only infested a few genotypes with a low alkaloid content. Nevertheless, the enhanced cultivation of such genotypes with a low alkaloid content under field conditions may lead to a high infestation with *A. pisum* and *A. craccivora*, respectively, due to the fact that aphids show phenotypic plasticity that allows them to exploit new host species and overcome novel resistance mechanisms in newly developed plant varieties (Cardoza et al., 2006). Studies of Cardoza et al. (2006) indicated that the ability to feed on narrow-leafed lupins is not characteristic for the species *M. persicae* but based on adaption. Their findings showed that one lupin-feeding clone from the Western Australia wheat belt, where extensive lupin cultivation is conducted, outperformed nine clones from eastern Australia with respect to infestation, where narrow-leafed lupins are rarely grown, on susceptible and resistant cultivars, respectively. Hence the authors concluded that the abundance of narrow-leafed lupins during the growing season would provide sufficient selection pressure for a lupin-adapted clone to predominate. These findings are indicating that the rearing plant is influencing the adaption of the aphid clones. Therefore, the slightly higher alkaloid content in the

variety Boregine used for rearing of *M. persicae* and *A. fabae,* compared with the breeding line Bo083521AR used for *A. pisum,* could be the reason for the better adaptation of *M. persicae* and *A. fabae* to a slightly higher alkaloid content. In addition, the lowest infestation rates in our experiments shown by the clone of *A. craccivora*, reared on faba bean, may be explained by the lack of adaption. Moreover, findings of (Cabrera-Brandt et al., 2015) on the multiplication and feeding behavior of different *M. persicae* clones on the *Prunus* species *P. davidiana* used as a source of resistance to pests and diseases in peach breeding programs showed intra-specific variation between the different clones of *M. persicae.* Based on these results the authors concluded that the *M. persicae* clones with better survival on *P. davidiana* may possess a feeding mechanism that permits them to overcome the resistance. Therefore, the influence of the clone used for aphid multiplication trials has to be taken into account.

Findings of Berlandier and Sweetingham (2003) based on the sweet *L. angustifolius* cultivar Kalya, which was less susceptible to feeding of *M. persicae*, *A. craccivora* and *A. kondoi* than two other genotypes investigated, e.g. Tallerack, are underlining the results of the current study. Therefore, it is likely that not only the overall alkaloid content is influencing the aphids' host-plant acceptance, but also the alkaloid composition has an impact. In addition to Kalya the sweet cultivars Bora and Borlu revealed to be resistant to the multiplication of all aphids investigated with the exception of *M. albifrons* (Table 2.4-3). Quinolizidine alkaloids, present in *L. angustifolius,* are synthesized in the leaf chloroplast (Harborne et al., 1999) transported via the phloem to the different plant organs (Wink and Witte, 1984) and stored in epidermal cells and seeds (Wink, 1986, 1987b). Wink et al. (1995) showed that the alkaloid composition differs between *Lupinus* species, and for *L. angustifolius* leaves lupanine (40%), 13-hydroxylupanine (20%) and angustifoline (20%) were found to be the major alkaloids. In the present study the highest amount was determined for 13-hydroxylupanine with an average amount of 44.5%, followed by angustifoline (13.6%) and lupanine (8.1%).

Quinolizidine alkaloids are synthesized from the amino acid lysine via cadaverine (Hirai et al., 2000). Cadaverine is the precursor of cyclic alkaloids, e.g. lupanine, multiflorine and sparteine in *L. angustifolius* or lupinine in *L. luteus* (Suzuki et al., 1994; Aniszewski, 2007; Bunsupa et al., 2012). These cyclic alkaloids are subsequently transformed by enzymes through dehydrogenation, oxygenation or esterification (Suzuki et al., 1994; Hirai et al., 2000). Therefore, lupanine is the precursor for 13-hydroxylupanine which can be transformed to several esters like 13-tigloyloxylupanine, 13-benzoyloxylupanine, 13-cis-cinnamoyloxy-lupanine and 13-trans-cinnamoyloxylupanine, or to angustifoline and tetrahydrorhombifoline (Suzuki et al., 1994; Hirai et al., 2000). Wink et al. (1995) noted that the alkaloid profiles of lupin leaves are more diverse than of seeds and that esters

of 13-hydroxylupanine are mainly present in leaves, while the hydroxylated form is predominant in seeds. In the present study these esters had an average amount of 24.7% of the total alkaloid content in the leaves.

Results of the multiple regression showed that especially 13-tigloyloxylupanine plays an important role for reduced susceptibility to *A. fabae*, *A. pisum*, *A. craccivora* and *M. persicae*. It was described by Suzuki et al. (1994) and Okada et al. (2005) that the enzyme l3α-hydroxylupanine-0-tigloyltransferase is responsible for the transformation of 13-hydroxylupanine to 13-tigloyloxylupanine. Hirai et al. (2000) and Saito et al. (1993) found, that in sweet and bitter genotypes of *L. angustifolius, L. albus* and *L. luteus* the same amount of l3α-hydroxylupanine-0-tigloyltransferase activity was detected. Based on these results (Saito et al., 1993) suggested that the general pathway of alkaloid synthesis is blocked in sweet plants, not a specific step in the formation of single alkaloids. Due to the fact that the level of the amino acid lysine and the decarboxylated intermediate cadaverine was not differing between sweet and bitter genotypes, they concluded that the limiting step for quinolizidine alkaloid biosynthesis of alkaloid poor genotypes is the enzymatic reaction from cadaverine to the first cyclic alkaloids. Furthermore, they suggested that the genes encoding enzymes for the initiation of cyclic alkaloid formation are controlled independently from genes encoding enzymes for later steps, e.g. l3α-hydroxylupanine-0-tigloyltransferase. Therefore, the alkaloid synthesis should not be completely down regulated to preserve a sufficient 13-tigloyloxylupanine content leading to a reduced susceptibility against aphids.

The findings of the multiple regression, indicating that 13-tigloyloxylupanine is playing an important role in aphid susceptibility, should be included in the breeding process, i.e. breeding genotypes with a low alkaloid content but a relatively high 13-tigloyloxylupanine content in order to combine the low alkaloid content needed for food and feed with a reduced level of aphid susceptibility. The content of 13-tigloyloxylupanine may be used as an indirect selection criterion, as it is very difficult to include rearing of aphids and determination of the multiplication rate into practical lupin breeding.

3. Feeding behavior of aphids on narrow-leafed lupin (*Lupinus angustifolius*) genotypes varying in the content of quinolizidine alkaloids.

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3.1. Abstract

Since the beginning of breeding narrow-leafed lupins [*Lupinus angustifolius* L. (Fabaceae)] with a low alkaloid content, susceptibility to several aphid species has increased. Therefore, the probing and feeding behavior of *Aphis fabae* Scopoli, *Aphis craccivora* Koch, *Acyrthosiphon pisum* (Harris), *Myzus persicae* (Sulzer), and the well-adapted *Macrosiphum albifrons* Essig (all Hemiptera: Aphididae) was studied over 12 h on narrow-leafed lupin genotypes containing varying amounts and compositions of alkaloids. We used the electrical penetration graph (EPG) technique to obtain information on the influence of alkaloid content and composition on the susceptibility to various aphid species. Results indicated that the total time of probing of *A. fabae*, *A. craccivora*, *A. pisum*, and *M. persicae* increased with a reduced alkaloid content, whereas the alkaloid content had no influence on *M. albifrons*. Almost all of the individuals (>93%) conducted sieve element phases on the highly susceptible genotype Bo083521AR (low alkaloid content). A reduced occurrence of phloem phases was observed during the 12-h recording on the alkaloid-rich cultivar Azuro, especially for *A. pisum* (37.5%) and *A. fabae* (55.0%). Furthermore, aphids feeding on genotypes with low alkaloid content had in most cases significantly longer sieve element phases than when feeding on resistant genotypes (Kalya: low alkaloid content, yet resistant; Azuro: high alkaloid content, resistant), whereas *M. albifrons* showed the longest phloem phase on the alkaloid-rich cultivar Azuro. As most significant differences were found in phloem-related parameters, it is likely that the most important plant factors influencing aphid probing and feeding behavior are localized in the sieve elements. The aphids' feeding behavior on the cultivar

Kalya, with a low alkaloid content but reduced susceptibility, indicates that not only the total alkaloid content influences the feeding behavior but additional plant factors have an impact.

3.2. Introduction

The genus *Lupinus* (Fabaceae) is indigenous to the Americas as well as to the Mediterranean region (Adhikari et al., 2012) and comprises more than 400 species of which four are of agronomic interest: *Lupinus angustifolius* L. (narrow-leafed lupin), *Lupinus albus* L. (white lupin), *Lupinus luteus* L. (yellow lupin), and *Lupinus mutabilis* Sweet (Andean lupin) (Reinhard et al., 2006). In 2012, an area of 887 014 ha of lupins was harvested worldwide, resulting in a total production of 1.29 million tons (FAOSTAT, 2014). Due to high yield losses caused by the fungal disease anthracnose [*Colletotrichum lupini* (Bondar) Nirenberg, Feiler & Hagedorn (Damm et al., 2012)] in traditional lupin-growing countries such as Poland, Portugal, Russia, France, and Germany, the narrow- leafed lupin (*L. angustifolius*) has gained more importance because of the availability of anthracnose resistant cultivars (Adhikari et al., 2011). Despite the occurrence of anthracnose, the cultivation of lupins, particularly of the narrow-leafed lupin, is of growing interest because of its high protein content. As nitrogen-fixing plants, lupins are of special interest for organic farming, where the utilization of nitrogen fertilizer is prohibited (Jensen et al., 2004). Its contribution of fixed nitrogen and organic matter to soil results in increased yields of successive crops and has therefore excellent potential for sustainable crop-rotation systems (Kurlovich, 2002; Ksiazkiewicz et al., 2013). Because of their high protein content of 27–40% (Jansen et al., 2013), the use of narrow- leafed lupins in human and animal nutrition is of great interest. However, wild lupin species produce a high level of quinolizidine alkaloids to protect themselves from herbivores (Wink, 1998). Lupin alkaloids are produced in leaf chloroplasts, then distributed all over the plant via the phloem and stored in epidermal cells and in seeds (Wink and Witte, 1984; Wink et al., 1995). Field trials showed that the total alkaloid content in seeds is higher than in leaves but a high correlation between seed and leaf content was detectable (J Philippi, E Schliephake, HU Jürgens, and G Jansen, unpubl.). Only very specialized species such as the lupin aphid, *Macrosiphum albifrons* Essig (Hemiptera: Aphididae), are able to use lupins as host plants. Quinolizidine alkaloids are toxic for animals (including humans), and intoxications cause convulsions, trembling, and death from respiratory and cardial arrest (Ganzera et al., 2010). These difficulties in the use of lupins for food or feed have led to breeding of

so-called sweet lupins, with a maximum of 0.05% alkaloids in the seeds (Fischer and Sengbusch, 1935; Sengbusch, 1942). Today, a threshold of 0.05% for animal feed and 0.02% for human nutrition is generally accepted (Jansen et al., 2009). As a result of the reduced alkaloid content in sweet lupins, a high susceptibility to insect herbivores and plant pathogens was observed (Wink, 1985, 1988; Wink and Witte, 1991). In Australia, which is one of the major lupin growing areas, aphids are an important pest, causing severe yield losses by feeding (Berlandier and Sweetingham, 2003) and virus transmission (Thackray et al., 2004). Yield loss by feeding was also observed in field trials in Germany. The parthenogenetic reproduction and viviparity of aphids leads to short generation times and high rates of reproduction, as a result of which even a small initial infestation usually results in large populations and significant plant damage (Webster et al., 2008). Considering the currently discussed climate change, an increase in the number of generations of Aphidoidea is predicted (Yamamura and Kiritani, 1998), resulting in a presumably higher importance of aphid infestation in lupin cultivation also in northern growing regions. To avoid yield losses, aphids may be controlled by insecticides, but spraying results in additional costs and is harmful to the environment. In addition, insecticides are prohibited in organic farming, where lupin cultivation is of special interest. For these reasons, aphid resistance is an important aspect in breeding sweet lupin cultivars.

Several aphid species are known to infest lupins (Holman, 2009) and may cause yield losses up to 100% by feeding (Kordan et al., 2008). *Macrosiphum albifrons* is well-adapted to lupins, particularly to lupins with a high alkaloid content, and causes considerable infestations (Ferguson, 1994). It was observed that *M. albifrons*, feeding on alkaloid-rich lupins, is able to store alkaloids and utilize these as a protection against predators, such as the carabid *Carabus problematicus* Herbst (Wink and Roemer, 1986) or the sevenspotted lady beetle *Coccinella septempunctata* L. (Gruppe and Roemer, 1988). As recorded by Wink and Roemer (1986) alkaloids serve the lupin aphid as a cue to find suitable host plants, whereas plants with low alkaloid content seem to be less attractive. On lupins with a reduced alkaloid content, the black bean aphid, *Aphis fabae* Scopoli, as a generalist and the pea aphid, *Acyrthosiphon pisum* (Harris) (both Hemiptera: Aphididae), as a specialist on different legume species are important, but they are not able to colonize alkaloid-rich lupins (Gruppe and Roemer, 1988; Wink and Witte, 1991; Kordan et al., 2008; Kordan et al., 2012). In addition, the polyphagous green peach aphid, *Myzus persicae* (Sulzer) (Hemiptera: Aphididae), feeding on plants from more than 40 families, has the potential to infest sweet lupins at a high rate (Edwards, 2001). Furthermore, the cowpea aphid, *Aphis craccivora* Koch (Hemiptera: Aphididae), can colonize sweet lupins. This aphid species is ubiquitous in lupin-growing areas in

Australia, causing severe yield losses (Zehnder et al., 2001).

Previous investigations (Zehnder et al., 2001; Berlandier and Sweetingham, 2003; Adhikari et al., 2012) assumed aphid resistance in the narrow-leafed lupin cv. Kalya, despite of its low alkaloid content. Zehnder et al. (2001) suggested that feeding-deterrent compounds localized in the phloem are a component of cowpea aphid resistance in cv. Kalya, based on the observation that cowpea aphids spent a reduced time in sieve element phases compared to a susceptible cultivar. However, no further information about this resistance is available. Therefore, to obtain detailed information on aphid resistance in sweet lupins, the electrical penetration graph (EPG) technique was used to investigate the penetration and feeding behavior of *M. albifrons*, *M. persicae*, *A. fabae*, *A. craccivora*, and *A. pisum* on various genotypes of narrow-leafed lupins, differing in leaf alkaloid content and composition.

3.3. Materials and methods

Aphids

Macrosiphum albifrons, *A. fabae*, *A. craccivora*, *M. persicae*, and *A. pisum* have been cultivated since several years in the greenhouse at the Julius Kühn-Institute in Quedlinburg, Germany. Aphid rearing was carried out in Plexiglas cages (50 x 60 x 65 cm) in the greenhouse, under longday conditions (16 h of light using high-pressure sodium lamps). *Aphis fabae*, *M. albifrons*, and *M. persicae* were reared on *L. angustifolius* cv. Boregine, *A. pisum* on *L. angustifolius* breeding strain Bo083521AR, and *A. craccivora* on fava bean, *Vicia faba* cv. Scirocco (Fabaceae).

Plant material

Plants of *L. angustifolius* cv. Azuro (high alkaloid content), breeding strain Bo083521AR (Saatzucht Steinach, Steinach, Germany), cv. Boregine, and cv. Kalya (all sweet lupins), were cultivated under controlled greenhouse conditions at L16 (20 °C) : D8 (18 °C) without any pesticide treatments. Plants were grown in pots (8 x 8 x 8 cm) in standard soil (Einheitserde Classic Profi Substrat CL T SM Sandfein; Einheitserdewerke, Sinntal, Germany). Plants in BBCH stage 25–31 (Dracup and Kirby, 1996) were used for EPG analysis.

Analysis of alkaloid content

Leaves of 10 plants [BBCH stage 31–35 (Dracup and Kirby, 1996)] per genotype were sampled in two replications. The leaves were frozen in liquid nitrogen and stored at -80 °C for at least 72 h. Subsequently, the samples were lyophilized for approximately 24 h

and stored at room temperature until analysis, according to the method described by Torres et al. (2002)with some modifications. The lyophilized leaves were ground with the variable speed rotor mill ‘Pulverisette 14’ (Fritsch, Idar- Oberstein, Germany). A sample of 0.5 g for sweet genotypes and 0.05 g for genotypes with a high alkaloid level was blended with 10 ml 1N HCl and stirred for 1 h. Next, 50 µl caffeine solution (2 mg ml^{-1} methyl alcohol; Sigma-Aldrich, Steinheim, Germany) was added as internal standard for the subsequent quantification and the homogenate was centrifuged for 20 min at 10 000 g. The supernatant was adjusted to pH≥12 by adding 1 ml 25% ammonia and 1.7 ml 6N NaOH, and was applied to a glass extraction column with Hydromatrix (high purity, inert diatomaceous earth sorbent; Agilent Technologies, Waldbronn, Germany). Alkaloids were eluted with methylene chloride and the solvent was removed by evaporation. The residue was dissolved in 200 µl methyl alcohol and analyzed by a 7890A gas chromatograph (Agilent Technologies) with flame ionization detector. The alkaloids were separated on a capillary column DB-1 (25 m x 0.20 mm x 0.33 µm; Agilent J&W). Pure sparteine (Sigma-Aldrich), lupanine, and 13-hydroxylupanine (both provided by HU Jürgens, Julius Kühn- Institute, Groß Lüsewitz, Germany) were used for calibration and quantification, with caffeine as internal standard. For the quantification of all other alkaloids, the lupanine calibration curve was applied. Alkaloid identification was performed by mass spectrometry (MSD 5975C; Agilent Technologies), by comparing the mass spectra and Kovats retention indices with data from Wink et al. (1995).

<u>EPG recording</u>

Experiments were conducted at room temperature (18–22 °C) with ceiling-mounted fluorescent tubes for illumination. Apterous female aphids were collected from colonies on the respective rearing plants by using a damped marten-hair brush (size 0) and detained in a Petri dish for 1 h of starving (Diaz-Montano et al., 2007). During this period, the aphids were fixed by vacuum on a small hole in a plastic disc (Schliephake et al., 2013) and a 2 to 2.5-cm-long thin gold wire (17 µm diameter) was attached to the back of their abdomen using an organic, water-soluble silver glue (Tjallingii, 1978). The opposite end of the gold wire was attached to a copper wire electrode soldered to a brass nail, serving as aphid electrode, which was placed in the input of the first stage amplifier. A second electrode (copper, ca. 10 cm long) was inserted in the soil of the potted plants. The aphid attached to the electrode was fixed approximately 0.5 cm above the newly expanded leaves, clamped with the abaxial leaf side face up as aphids prefer settling on the lower surface of the leaf (Pettersson et al., 2007). Both electrodes were connected to an 8- channel Giga-8 DC EPG amplifier with 109 Ω input resistance and an analog-digital conversion rate of 100 Hz (EPG Systems, Wageningen, The Netherlands). The

EPG recording experiments were conducted in a Faraday cage to avoid electrical noise signals. The aphids were placed on the leaf surface directly after starting the data acquisition program 'Stylet + d' (EPG Systems) and remained for a recording period of 12 h. An adjustment of the voltage source was carried out as described by Tjallingii (2006), so that the amplifier output signal was between +5 and –5 V, with positive values in intercellular positions and negative values in intracellular positions of stylet tips. Data from aphids dead or disconnected at the end of the run were discarded. For each combination of aphid and genotype, at least 18 replications were conducted (Table 3.3-1).

Table 3.3-1: Number of electrical penetration graph (EPG) runs of surviving aphids of five species on high- (Azuro) and low-alkaloid-level genotypes (Kalya, Boregine, and Bo083521AR) of *Lupinus angustifolius* with successful tether connection of 12 h and number of analyzed runs with penetration signal (ps) during experimental time and Jacknife Distance (JD) <50 in the outlier analysis, finally used for EPG parameter analysis

Aphid	Number of	Azuro	Kalya	Boregine	Bo083521AR
A. fabae	Runs (12 h)	22	26	21	26
	Analyzed runs	20	19	19	20
A. pisum	Runs (12 h)	25	22	26	20
	Analyzed runs	24	21	25	18
M. persicae	Runs (12 h)	20	22	32	23
	Analyzed runs	19	21	21	22
A. craccivora	Runs (12 h)	24	27	21	23
	Analyzed runs	23	25	21	22
M. albifrons	Runs (12 h)	31	23	22	21
	Analyzed runs	27	18	19	21

No randomization of treatments was conducted, i.e., one lupin genotype was tested by one aphid species at a time. EPG parameters EPG data were analyzed with the 'Stylet + a' software (EPG Systems) with regard to the specific waveforms C (stylet movement; stylet is in contact with plant tissue and performing different pathway activities including potential drops, indicating that the stylet is puncturing the cell), E1 (phloem salivation phase; release of saliva in the phloem), E2 (phloem feeding phase; ingestion of phloem sap), F (derailed stylet mechanics; difficulties during penetration process; no ingestion), G (xylem feeding phase; ingestion from xylem) described by Tjallingii (1978, 1994). Probing (Pr) includes all of the previously stated waveforms, non-probing is defined as the phase where no contact between stylet and plant tissue is recorded. Pathway is defined as the part of probing during which there is neither phloem nor xylem activity. Subsequently, several sequential (time measured from a certain point, e.g., time from

the first E1 to the first E2) and non-sequential parameters (e.g., numbers, sums, or durations of waveforms) were calculated based on the waveform data (list of standardized EPG-variables on www.epgsystems.eu). For this purpose, an Excel-VBA macro according to the method of Schliephake et al. (2013) was used, facilitating the stepwise analysis of total probing time by hours. In summary, 39 parameters were used for statistical analysis of principal components and for factor analysis (FA) (Table 3.4-1).

Statistical analysis

The statistical analysis of the EPG data was conducted with SAS version 9.3 (SAS Institute, Cary, NC, USA). Because the data of the recorded EPG parameters were not normally distributed, the procedure NPAR1WAY was applied for nonparametric statistical analysis using the Kruskal–Wallis test, and a pairwise comparison was conducted to find significant differences between genotypes. The alkaloid composition was analyzed with Proc GLM followed by a Tukey test. To fulfill the requirement of normal distribution of the residuals, a $\sqrt{x}$-transformation was conducted. For the analysis of Spearman's correlation coefficient the Proc CORR application was used, correlating the mean alkaloid content in the leaves with the single values of the parameters. To eliminate outliers, an outlier analysis based on Jackknife distances was conducted by using the multivariate methods option. Runs showing a Jackknife distance >50 were not taken into account for analysis (Table 3.3-1). Because of the correlation between many EPG parameters, a principal component analysis (PCA) was conducted to reduce the variability of parameters. Subsequently, a FA was calculated to identify the parameter groups explaining most of the variability observed. For Outlier Analysis, PCA, and FA the JMP_ Genomics 5.1 software (SAS Institute) was used.

3.4. Results

Analysis of quinolizidine alkaloid content

The total alkaloid content of the cv. Azuro was 299 higher than that of cv. Kalya, 419 higher than that of cv. Boregine, and 1609 higher than that of Bo083521AR (Table 3.4-2). 13-hydroxylupanine was the major alkaloid in all genotypes investigated. The composition of the analyzed alkaloids differed between genotypes. For Boregine and Bo083521AR, 13-cis-cinnamoyloxylupanine was not detectable, and no 13-transcinnamoyloxylupanine, tetrahydrorhombifoline, and angustifoline was detected in the leaves of Bo083521AR. The alkaloid sparteine, known from leaves (Vilariño et al., 2005) and seeds (Wink et al., 1995) of other narrow- leafed lupin genotypes, was found only in traces in our set of cultivars.

Table 3.4-1: List of parameters used for the principal component analysis (PCA) and factor analysis (FA)

	Parameter	Definition	Sequential (s)/ non-seq. (ns)
Non-probing	n_Np	No. non-probing (Np) periods	Ns
	a_Np	Average of non-probing (Np) periods	Ns
	m_Np	Median of non-probing (Np) periods	Ns
	s_Np	Sum of of non-probing (Np) periods	Ns
Probing	n_Pr	No. probes (Pr)	Ns
	a_Pr	Average of probes (Pr)	Ns
	m_Pr	Median of probes (Pr)	Ns
	s_Pr	Sum of probes (Pr)	Ns
	n_bPr	No. brief probes (bpr)	Ns
	t_1Pr	Time to first probe (Pr)	S
	d_1Pr	Duration of first probe (Pr)	S
Pathway	n_C	No. C periods (including A, B, C and potential drops (pd), but not F and G)	Ns
	a_C	Average duration C period	Ns
	m_C	Median duration C period	Ns
	s_C	Sum of C periods	Ns
Derailed stylet Mechanics	n_F	No. F periods	Ns
	s_F	Sum of F periods	Ns
Xylem	n_G	No. G periods	Ns
	s_G	Sum of G periods	Ns
	nPr_1G	No. probes before first G period	Ns
	t_1G	Time to the first G period	S
Phloem	n_sgE1	No. single e1periods (only E1 without a preceding or subsequent E2 period)	Ns
	s_sgE1	Sum of single e1periods (only E1 without a preceding or subsequent E2 period)	Ns
	n_frE1	No. E1 fraction periods (only e1with a preceding or subsequent E2 period)	Ns
	s_frE1	Sum of E1 fractions (only e1with a preceding or subsequent E2 period)	Ns
	n_E1	No. E1 periods	Ns
	s_E1	Sum of E1 periods	Ns
	t_1E1	Time to first E1 period	S
	n_E12	No. E12 periods	Ns
	s_E12	Sum of E12 periods	Ns
	t_1E12	Time to the first E12 period	S
	n_E2	No. E2 periods	Ns
	s_E2	Sum of E2 periods	Ns
	t_1E2	Time to the first e2period	S
	n_sE2	No. sustainable E2 periods (>10 min)	Ns
	s_sE2	Sum of sustainable E2 periods (>10 min)	Ns
	t_1sE2	Time to firsts E2 period	S
	t_1E1_1E2	Time from the first E1 to the first E2 period	S
	t_1E1_1sE2	Time from the first E1 to the first sustainable E2 period (>10 min)	S

Table 3.4-2: Mean (±SD) alkaloid composition (µg g^{-1} dry leaf matter) in leaves of four *Lupinus angustifolius* genotypes analyzed by GC-MS

Alkaloid	Azuro	Kalya	Boregine	Bo083521AR	P
13-Hydroxy-lupanine	5066.9 ± 1268.0a	154.1 ± 77.4b	105.3 ± 83.0b	22.9 ± 2.2b	0.0006
13-Tigloyloxy-lupanine	945.9 ± 427.6a	62.5 ± 12.9b	44.1 ± 2.5b	7.9 ± 11.2b	0.0056
13-Benzoyloxy-lupanine	244.3 ± 111.1a	20.7 ± 29.3ab	21.2 ± 0.6ab	8.4 ± 11.9b	0.033
13-cis-Cinnamoyl-oxylupanine	677.9 ± 468.5a	10.9 ± 15.4b	0.0 ± 0.0b	0.0 ± 0.0b	0.018
13-trans-Cinnamoyl-oxylupanine	576.5 ± 426.4a	18.4 ± 26.0ab	8.6 ± 12.1ab	0.0 ± 0.0b	0.034
Lupanine	1708.2 ± 676.1a	33.5 ± 1.3b	22.3 ± 4.7b	14.7 ± 1.3b	0.0021
Angustifoline	1074.0 ± 439.2a	22.9 ± 10.3b	16.2 ± 3.3b	0.0 ± 0.0b	0.0023
Isolupanine	355.4 ± 89.2a	29.8 ± 3.8b	31.4 ± 6.6b	13.8 ± 0.3b	0.0008
Tetrahydro-rhombifoline	163.3 ± 9.5a	16.7 ± 3.1b	14.4 ± 0.5b	0.0 ± 0.0 (c)	<0.0001
Total	10812.3 ± 3915.6a	369.4 ± 179.4b	263.4 ± 113.3b	67.7 ± 26.2b	0.0019

Means within a row followed by different letters are significantly different (Tukey test: $P<0.05$)

EPG probing and feeding behavior

Proportions of feeding parameters: The mean duration time of probing (Pr) of *A. craccivora*, *A. fabae*, *A. pisum*, and *M. persicae* was reduced on the high alkaloid genotype Azuro (Figure 3.4-1) with the tendency to increase with reduced alkaloid content. The probing duration of *M. albifrons* was not affected by alkaloid content. For *A. fabae*, *A. pisum*, *M. persicae*, and *A. craccivora*, a shorter pathway (C) duration on the sweet breeding line Bo083521AR than on the bitter genotype Azuro was observed. For these aphids, the extension of the probing time was mainly caused by longer phloem feeding on the genotypes with a reduced alkaloid content. In contrast, the pathway duration of *M. albifrons* slightly increased with decreasing alkaloid content. Additionally, the longest phloem feeding period (E2) of *M. albifrons* was observed on the alkaloid-rich cv. Azuro. The percentage of aphids performing complete phloem feeding periods (E12; periods with both E1 and E2) during the experimental time was reduced on cv. Azuro for all aphid species except *M. albifrons* (Figure 3.4-2). Especially for *A. pisum* (37.5%) and *A. fabae* (55.0%), the number of aphids penetrating the phloem of Azuro plants was conspicuously reduced in comparison to the sweet genotypes.

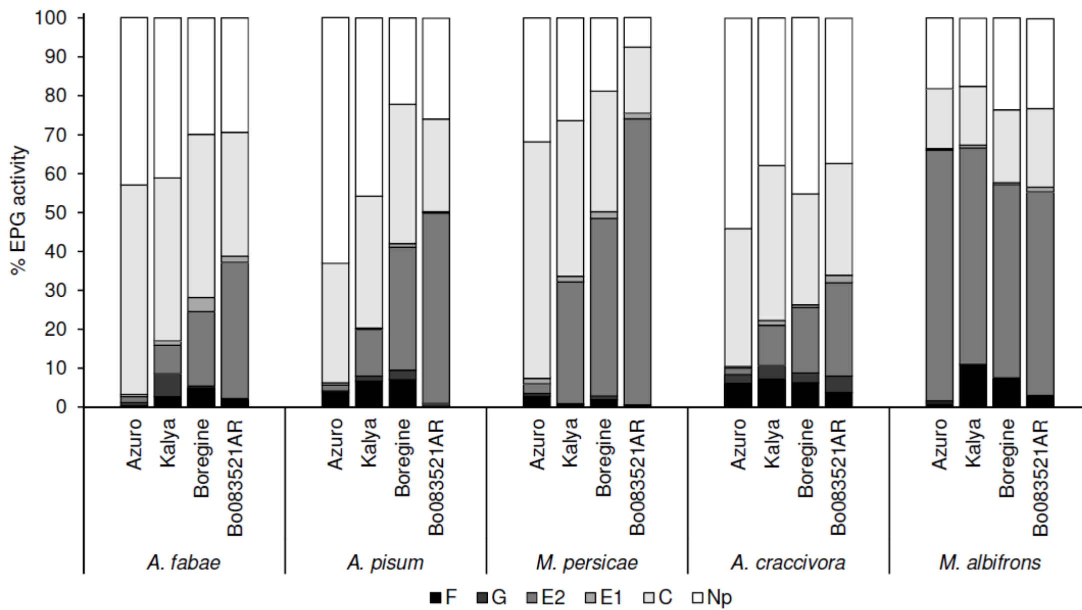

Figure 3.4-1: Mean electrical penetration graph (EPG) activity (%) of *Aphis fabae*, *Acyrthosiphon pisum*, *Myzus persicae*, *Aphis craccivora*, and *Macrosiphum albifrons* high- (Azuro) and low-alkaloid containing genotypes (Kalya, Boregine, and Bo083521AR) of *Lupinus angustifolius* during 12 h of observation with waveforms C (pathway), E1 (phloem salivation), E2 (phloem feeding), F (derailed stylet mechanics), and G (xylem feeding). Np, non-probing.

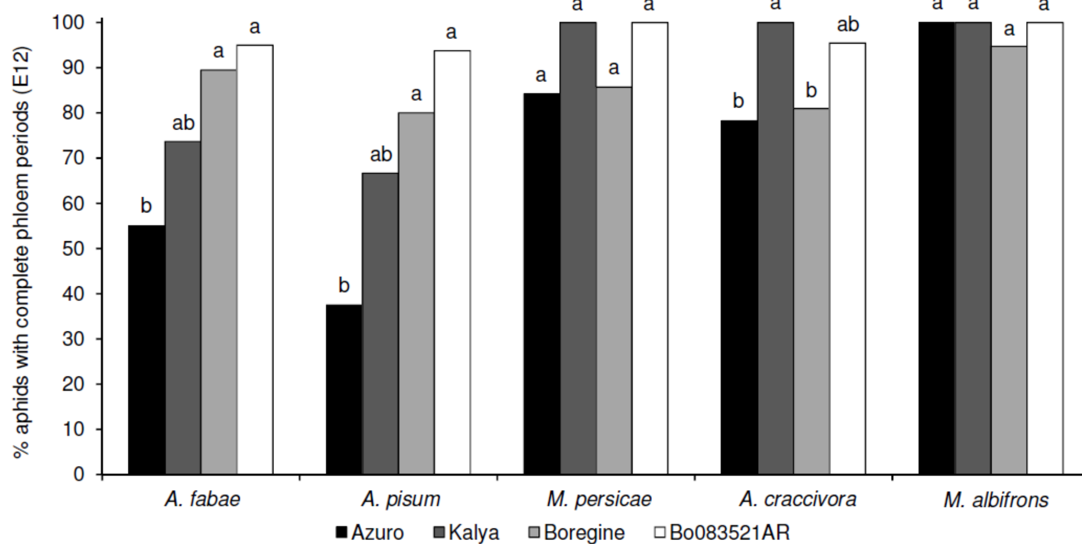

Figure 3.4-2: Percentage of *Aphis fabae*, *Acyrthosiphon pisum*, *Myzus persicae*, *Aphis craccivora*, and *Macrosiphum albifrons* on high- (Azuro) and low-alkaloid-level genotypes (Kalya, Boregine, and Bo083521AR) of *L. angustifolius* with complete phloemperiods (E12; periods with both E1 and E2) during 12 h of observation. Bars within an aphid species capped with different letters are significantly different (Kruskal–Wallis test: P<0.05).

Principal component analysis and factor analysis: The scatter plot of the first and second principal components of the PCA of Azuro (Figure 3.4-3 A) showed a partial separation of the *M. albifrons* individuals, whereas for the other aphids no separation was observed. These results indicate that the probing and feeding behavior of *M. albifrons* on Azuro differs from the behavior of the other aphid species investigated. In contrast, for the breeding line Bo083521AR no differences in the PCA of the probing and feeding behavior were observed (Figure 3.4-3 B). Results from the FA implied that the most important parameters influencing the first factor were the phloem feeding-related parameters total time of phloem feeding (s_E2), complete phloem periods (s_E12; periods with both E1 and E2), and sustained phloem feeding (s_sE2; phloem feeding periods longer than 10 min), all with negative factor values. The factor 2 was highly influenced by the number of phloem salivation periods (n_E1) and fractions (n_frE1), the number of phloem feeding (n_E2), and complete phloem periods (n_E12), for which positive factor values were observed. For the third factor the average duration of pathway (a_C; negative factor value) and number of non-probing periods (n_Np; positive factor value) were characteristic (data not shown).

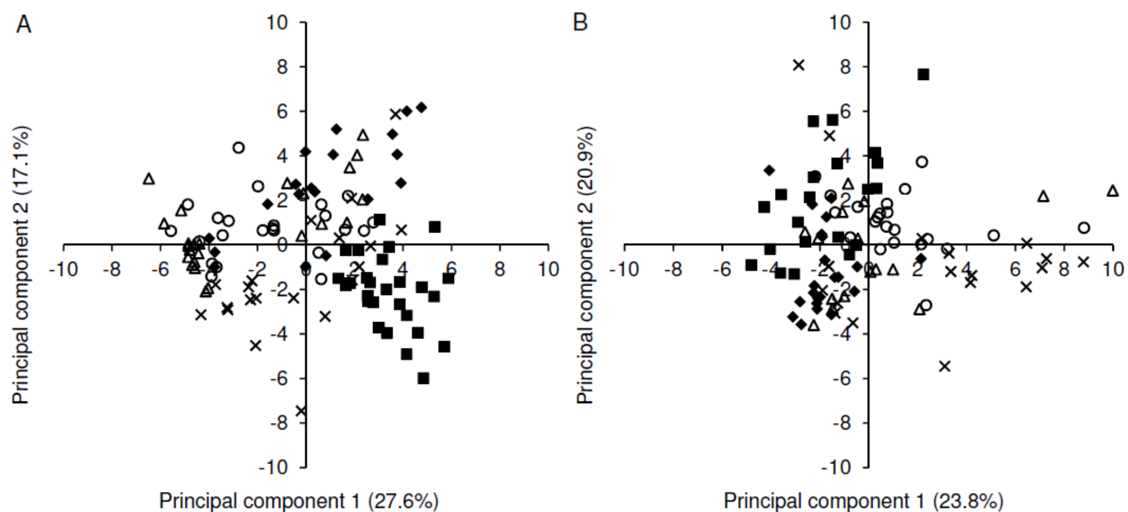

Figure 3.4-3: Principal component analysis (PCA) with the electrical penetration graph (EPG) parameters for x = *Aphis fabae*, ∆ = *Acyrthosiphon pisum*, ♦ = *Myzus persicae*, ○ = *Aphis craccivora*, and ■ = *Macrosiphum albifrons* on (A) cultivar Azuro and (B) the breeding line Bo083521AR.

Correlation between alkaloid content and EPG parameters.

The Spearman correlation analysis (correlation coefficient r_s) between the mean total alkaloid content and the single values of the different EPG parameters of *A. craccivora*, *A. fabae*, *A. pisum* and *M. persicae* (Table 3.4-3) revealed a high negative correlation to

several parameters, especially for the phloem feeding related parameters (s_E2, s_E12, s_sE2), which had a high influence on the first factor of the FA. In contrast, the data for *M. albifrons* gave a lower but positive correlation for these parameters. The number of parameters significantly correlated with the alkaloid content was higher for *M. persicae*, *A. pisum*, and *A. craccivora* than for *A. fabae*. Probing parameters (s_Pr, n_Pr) of *A. craccivora*, *A. pisum*, and *M. persicae* were highly correlated with the alkaloid content, whereas for *A. fabae* and *M. albifrons* no significant correlation was observed. Moreover, no genotype-specific differences were found for *A. fabae* and *M. albifrons* in these parameters (Table 3.4-4).

Probing parameters.

The time from the beginning of the recording to the first probe (t_1Pr) of *M. persicae* was affected by the genotype, with a significantly longer time to the first probe on Azuro (6.6 min) and Bo083521AR (6.7 min) than on Boregine (3.1 min) and Kalya (4.9 min). For *A. fabae*, *A. pisum*, *A. craccivora*, and *M. albifrons*, no significant differences between the genotypes were observable. Concerning the duration of the first probe (d_1Pr), no significant differences between the genotypes for all aphid species were observed. The total time of pathway (s_C) of *A. fabae*, *M. persicae*, and *A. craccivora* was reduced with a decreasing alkaloid content of the genotypes (r_s = 0.304 - 0.739). For *M. albifrons*, no significant differences between the genotypes were detected, but the tendency of an increasing pathway time with a decreasing alkaloid content was obvious, reflected by the correlation coefficient of r_s = -0.219. The number of pathway periods (n_C) carried out by *A. pisum*, *M. persicae*, and *A. craccivora* was higher on the alkaloid-rich genotype Azuro (50.2 - 56.0) and decreased with a reduced alkaloid content to 14.0 - 24.4 (r_s = 0.439 - 0.665), whereas no differences between the genotypes were detected for *M. albifrons* and *A. fabae*. Comparing the phloem feeding behavior of the aphids on the various genotypes, the number of complete phloem periods including phloem salivation and feeding (n_E12) was not genotype specific for *A. fabae* (Table 3.4-5). The highest values were recorded for *A. pisum* on Boregine (7.4), for *M. persicae* (6.0) and *A. craccivora* (5.1) on Kalya, and for *M. albifrons* on Bo083521AR (4.4). The longest time of phloem salivation (s_E1) occurred for *A. fabae* (25.9 min) and *A. pisum* (6.4 min) on the genotype Boregine, *A. craccivora* (14 min) and *M. albifrons* (8.8 min) showed the longest time on Bo083521AR. The total time of phloem salivation was not significantly different between the genotypes for *M. persicae* and also for the number of phloem salivation periods (n_E1) of *A. fabae*. The number of phloem salivation periods (n_E1) carried out on the different genotypes was in most cases higher than the number of complete phloem feeding periods (n_E12). Regarding the time to the first phloem

salivation (t_1E1), significant differences between the genotypes were found for *A. pisum*, *M. persicae*, and *A. craccivora*, with the longest time on Azuro and the lowest values on Kalya for *A. craccivora* and on Bo083521AR for *A. pisum* and *M. persicae*, whereas *M. albifrons* needed the longest time to the first phloem salivation on Boregine (158.7 min) and a significantly reduced time to the first phloem salivation was needed on Bo083521AR (38.6 min). The total time of complete phloem periods (s_E12) of *A. fabae*, *A. pisum*, *M. persicae*, and *A. craccivora* increased with a decreasing alkaloid content. *Macrosiphum albifrons* showed no significant differences, but a tendency of an increasing time with increasing alkaloid content was observed (r_s = 0.231). As revealed by the temporal progress of phloem feeding during the experimental time (Figure 3.4-4). *M. albifrons* fed continuously on all the cultivars, whereas the other aphid species showed only a slight increase of phloem phases on Azuro (Figure 3.4-4 A). The duration of phloem feeding time increased on the cultivars with a reduced alkaloid content, so that on line Bo083521AR the feeding of *M. persicae* was longer than that of *M. albifrons* (Figure 3.4-4 B–D). The longest time to the first complete phloem period (t_1E12) was observed for *A. fabae* (417.6 min), *A. pisum* (501.5 min), *M. persicae* (285.5 min), and *A. craccivora* (397.1 min) on Azuro and for *M. albifrons* on Boregine (162.8 min). The number of phloem feeding periods (n_E2) was not influenced by the alkaloid content for *A. fabae* (Table 3.4-6). For *A. pisum* and *A. craccivora*, the lowest number of phloem feeding periods was observed on Azuro, and for *M. persicae* on Bo083521AR. The lowest numbers of sustained phloem feeding periods for *M. persicae*, *A. pisum* *A. craccivora*, and *A. fabae* were observed on Azuro. A comparison of the feeding behavior between the genotypes indicated that the numbers of phloem feeding (7.7) and sustained phloem feeding periods (5.6) of *A. pisum* were highest on Boregine. The highest numbers of E2 (6.3) and sE2-periods (4.1) of *M. persicae* were detected on cv. Kalya. For *A. craccivora*, the highest number of phloem feeding periods was observed on cv. Kalya (5.2), but the highest number of sustained phloem feeding periods occurred on Bo083521AR (2.2). *Macrosiphum albifrons* carried out the most periods of phloem (4.9) and sustained phloem feeding (4.0) on Bo083521AR. The total times of phloem feeding (s_E2) and sustained phloem feeding (s_sE2) of *A. fabae*, *A. pisum*, *M. persicae*, and *A. craccivora* were opposed to the gradient of the alkaloid content. For *M. albifrons*, no significant genotype-specific differences were observed for the total time of sustained phloem feeding, but the number of these periods was significantly higher on Bo083521AR (4.0) than on the other genotypes. Although the time to the first phloem feeding (t_1E2) of *A. fabae* showed no significant differences between the genotypes analyzed, the time to the first sustained phloem feeding (t_1sE2) on the alkaloid-rich genotype Azuro was significantly longer. The time to the first phloem feeding and

sustained phloem feeding of *A. pisum* was significantly reduced by a decreasing alkaloid content. The time to the first phloem feeding of *A. craccivora* was significantly longer on Azuro (398.2 min) than on Kalya (167.5 min) and Bo083521AR (195.7 min), whereas the time to the first sustained phloem feeding was significantly longer on Azuro than on the sweet genotypes. For *M. persicae*, the time to the first phloem feeding was significantly longer on Azuro (286.8 min) than on Kalya (146.9 min) and Bo083521AR (109.2 min), and a significantly longer time to the first sustained phloem feeding was observed for Azuro than on the sweet genotypes. *Macrosiphum albifrons* showed the longest time to the first E2 (163.8 min) and sE2 (175.1 min) on cv. Boregine, the shortest time was recorded on Bo083521AR (43.3 and 49 min) with intermediate values for Azuro (109.1 and 115.5 min) and Kalya (133.5 and 149.8 min). Regarding the parameters of the xylem phases, no genotype- specific differences were observed and a correlation between total alkaloid content and xylem activities was not detected for any aphid species investigated (data not shown).

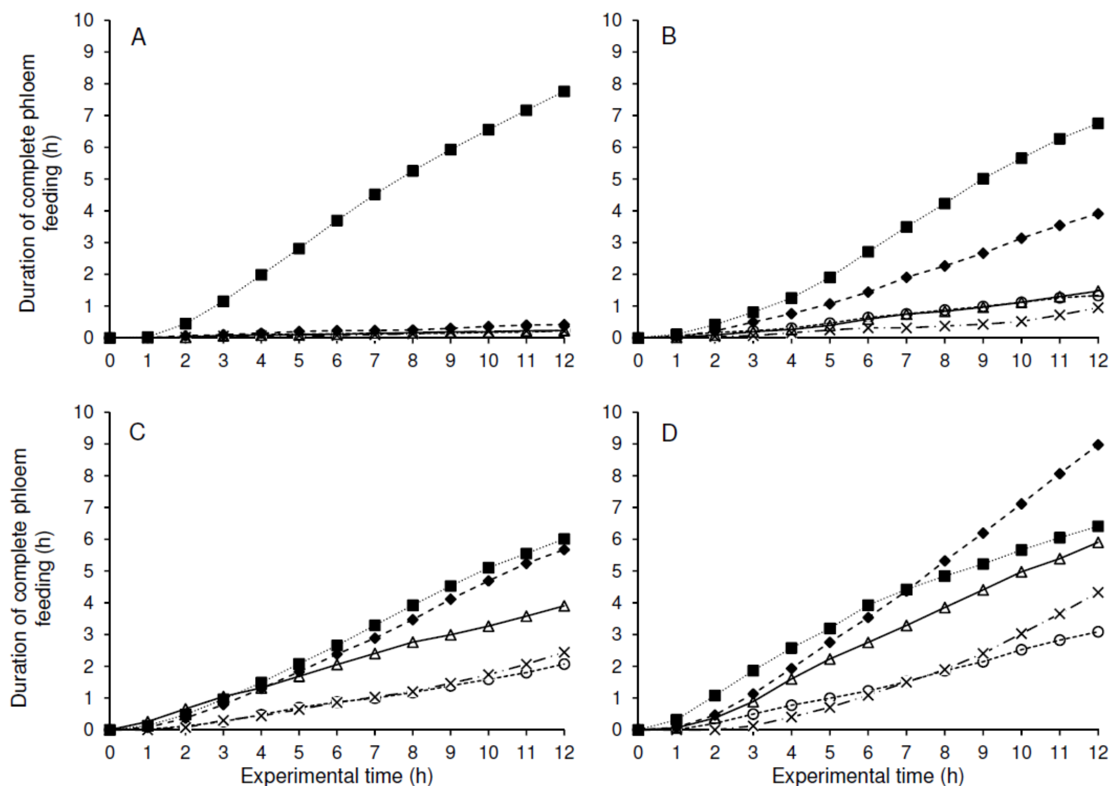

Figure 3.4-4: Total time of complete phloem periods (s_E12; periods with both, E1 and E2) of x = *Aphis fabae*, ∆ = *Acyrthosiphon pisum*, ♦ = *Myzus persicae*,○ = *Aphis craccivora*, and ■ = *Macrosiphum albifrons* on (A) Azuro (high alkaloid level), (B) Kalya, (C) Boregine, and (D) Bo083521AR (all three low alkaloid level).

Table 3.4-3: Spearman's correlation coefficient r_s (with P in parentheses) for the mean total alkaloid content and individual electrical penetration graph (EPG) parameter values of *Aphis fabae*, *Acyrthosiphon pisum*, *Myzus persicae*, *Aphis craccivora*, and *Macrosiphum albifrons*

Parameter	*A. fabae*	*A. pisum*	*M. persicae*	*A. craccivora*	*M. albifrons*
Total time (min) of probing (s_Pr)	-0.220 (0.054)	-0.577 (<0.0001)	-0.589 (<0.0001)	-0.253 (0.016)	0.124 (0.26)
No. probes (n_Pr)	0.085 (0.46)	0.492 (<0.0001)	0.631 (<0.0001)	0.484 (<0.0001)	-0.055 (0.62)
Duration (min) of the first probe (d_1Pr)	0.001 (1.0)	-0.017 (0.88)	-0.142 (0.20)	-0.077 (0.47)	0.025 (0.82)
Time (min) to the first probe (t_1Pr)	-0.055 (0.64)	0.009 (0.94)	-0.048 (0.66)	0.079 (0.46)	-0.089 (0.42)
Total time (min) of pathway (s_C)	0.328 (0.0034)	0.061 (0.58)	0.739 (<0.0001)	0.304 (0.0034)	-0.219 (0.046)
No. pathway periods (n_C)	0.054 (0.64)	0.439 (<0.0001)	0.665 (<0.0001)	0.507 (<0.0001)	-0.100 (0.37)
Total time (min) of phloem salivation (s_E1)	-0.294 (0.0090)	-0.193 (0.075)	0.049 (0.66)	-0.282 (0.0068)	-0.287 (0.0080)
No. phloem salivation periods (n_E1)	-0.144 (0.21)	-0.276 (0.010)	0.302 (0.0055)	-0.091 (0.39)	-0.318 (0.0032)
Time (min) from the first probe to first phloem salivation (t_1E1)	0.114 (0.32)	0.475 (<0.0001)	0.312 (0.0041)	0.220 (0.036)	0.389 (0.0003)
Total time (min) of complete phloem periods (s_E12)	-0.567 (<0.0001)	-0.645 (<0.0001)	-0.813 (<0.0001)	-0.589 (<0.0001)	0.231 (0.035)
No. complete phloem periods (n_E12)	-0.122 (0.29)	-0.302 (0.0047)	0.306 (0.0050)	-0.084 (0.43)	-0.202 (0.066)
Time (min) from first probe to first complete phloem periods (t_1E12)	0.097 (0.40)	0.463 (<0.0001)	0.316 (0.0036)	0.217 (0.039)	0.365 (0.0006)
Total time (min) of phloem feeding (s_E2)	-0.565 (<0.0001)	-0.651 (<0.0001)	-0.815 (<0.0001)	-0.576 (<0.0001)	0.245 (0.025)
No. phloem feeding periods (n_E2)	-0.144 (0.21)	-0.313 (0.0033)	0.292 (0.0074)	-0.105 (0.32)	-0.265 (0.015)
Time (min) from first probe to first phloem feeding (t_1E2)	0.088 (0.44)	0.463 (<0.0001)	0.301 (0.0057)	0.218 (0.038)	0.365 (0.0007)
Total time of sustained phloem feeding (>10 min) (s_sE2)	-0.604 (<0.0001)	-0.697 (<0.0001)	-0.834 (<0.0001)	-0.593 (<0.0001)	0.249 (0.022)
No. sustained phloem feeding periods (>10 min) (n_sE2)	-0.481 (<0.0001)	-0.538 (<0.0001)	-0.239 (0.030)	-0.474 (<0.0001)	-0.359 (0.0008)
Time from first probe to first sustained phloem feeding (>10 min) (t_1sE2)	0.330 (0.0032)	0.562 (<0.0001)	0.484 (<0.0001)	0.370 (0.0003)	0.339 (0.0016)

Table 3.4-4: Mean (±SD) electrical penetration graph (EPG) parameter values constituting the probing (Pr) and pathway phases (C) of *Aphis fabae*, *Acyrthosiphon pisum*, *Myzus persicae*, *Aphis craccivora*, and *Macrosiphum albifrons* on four genotypes of *Lupinus angustifolius* during 12 h experimental time

Parameter	Aphid	Azuro	Kalya	Boregine	Bo083521AR	P
No. probes (n_Pr)	*A. fabae*	13.8 ± 8.2a	13.5 ± 8.9a	15.3 ± 11.4a	11.3 ± 7.4a	0.66
	A. pisum	51.3 ± 25a	47.4 ± 28.5ab	32.1 ± 18.9b	20.9 ± 16.6c	0.0001
	M. persicae	50.1 ± 19.2a	25.9 ± 11.3b	28.7 ± 22.7b	12.3 ± 8c	<0.0001
	A. craccivora	46.7 ± 22.2a	26.8 ± 13.3b	24.6 ± 13.6bc	20.6 ± 10.9c	<0.0001
	M. albifrons	25 ± 16a	18.1 ± 14.8a	27.1 ± 24.2a	34.1 ± 30.1a	0.35
Total time (min) of probing (s_Pr)	*A. fabae*	410.6 ± 200.8a	423.9 ± 149.4a	504.4 ± 125.7a	508.5 ± 137.3a	0.20
	A. pisum	265.8 ± 157.2a	390.2 ± 171.5b	560.4 ± 152c	533.5 ± 161.7c	<0.0001
	M. persicae	490.9 ± 133.1a	529.9 ± 94.6ab	584.9 ± 116.9b	665.6 ± 40.5c	<0.0001
	A. craccivora	331.2 ± 112.2a	446.9 ± 89b	393.8 ± 184.8ab	451.5 ± 127.5b	0.0097
	M. albifrons	588.7 ± 74.6a	593.9 ± 104.8a	550.3 ± 140.7a	552.8 ± 110.7a	0.57
Time (min) to the first probe (t_1Pr)	*A. fabae*	61.4 ± 116.4a	82.1 ± 111.1a	32.1 ± 49.3a	60.3 ± 90a	0.47
	A. pisum	9 ± 16.5a	6.5 ± 9.9a	6.6 ± 10a	24 ± 54.4a	0.86
	M. persicae	6.6 ± 7.8a	4.9 ± 11.3b	3.1 ± 5b	6.7 ± 6.3a	0.0013
	A. craccivora	2.2 ± 1.7a	3.8 ± 5.4a	14.2 ± 24.6a	3.3 ± 8.7a	0.10
	M. albifrons	7.6 ± 9.2a	19.7 ± 31.8a	6 ± 6.2a	10.1 ± 9.9a	0.54
Duration (min) of the first probe (d_1Pr)	*A. fabae*	13.4 ± 21.1a	44.5 ± 89.4a	33.5 ± 83.9a	62.8 ± 160.7a	0.56
	A. pisum	6 ± 12a	16.1 ± 50.2a	35.8 ± 113.6a	10.1 ± 12.4a	0.087
	M. persicae	2.3 ± 4.5a	2.7 ± 6.9a	6.3 ± 14a	3.5 ± 5.1a	0.43
	A. craccivora	0.6 ± 0.4a	17.6 ± 47.3a	8.6 ± 20.7a	1.1 ± 2.4a	0.82
	M. albifrons	4.3 ± 8.5a	60.1 ± 161.6a	2.5 ± 4.1a	71.1 ± 163.1a	0.62
No. pathway periods (n_C)	*A. fabae*	16.6 ± 11.6a	16.5 ± 10a	19.8 ± 13a	13.7 ± 10.7a	0.29
	A. pisum	54 ± 24.4a	51.4 ± 27.6ab	40.5 ± 19.4b	22.9 ± 17.3c	0.0003
	M. persicae	56 ± 18.5a	32.2 ± 13.2b	32.4 ± 22.2b	14 ± 9.4c	<0.0001
	A. craccivora	50.2 ± 22a	35.8 ± 13.6b	34.8 ± 26.4bc	24.4 ± 11c	<0.0001
	M. albifrons	27.8 ± 16.9a	23.6 ± 15.7a	31.5 ± 25.3a	39.5 ± 30.9a	0.55
Total time (min) of pathway (s_C)	*A. fabae*	387.3 ± 200.3a	301.4 ± 112.8ab	301.8 ± 99.6ab	229.1 ± 149.8b	0.024
	A. pisum	221.3 ± 140.2a	244 ± 88.4a	258.1 ± 111.4a	171.4 ± 98a	0.15
	M. persicae	437.5 ± 113.7a	287.7 ± 68.7b	223.3 ± 121.6c	122.4 ± 85.8d	<0.0001
	A. craccivora	255.2 ± 85.7a	286.3 ± 79.8a	191.4 ± 96.9b	206.4 ± 77.4b	0.0008
	M. albifrons	111.2 ± 56.8a	108.6 ± 47.8a	134.5 ± 83.1a	145.7 ± 64.2a	0.19

Means within a row followed by different letters are significantly different (Kruskal-Wallis tests: P<0.05).

Table 3.4-5: Mean (±SD) electrical penetration graph (EPG) parameter values constituting the phloem salivation (E1) and complete phloem phases (E12) of *Aphis fabae*, *Acyrthosiphon pisum*, *Myzus persicae*, *Aphis craccivora*, and *Macrosiphum albifrons* on four genotypes of *Lupinus angustifolius* during 12 h experimental time

Parameter	Aphid	Azuro	Kalya	Boregine	Bo083521AR	P
No. phloem salivation periods (n_E1)	*A. fabae*	2.7 ± 3.9 a	2.7 ± 3.2 a	4.4 ± 3.4 a	3.1 ± 5.0 a	0.071
	A. pisum	2.3 ± 3.5 a	3.1 ± 3.2 a	8.0 ± 6.1 b	2.5 ± 2.1 a	0.0007
	M. persicae	5.4 ± 4.6 ab	6.8 ± 4.0 b	4.0 ± 3.1 a	2.8 ± 2.8 a	0.0042
	A. craccivora	2.5 ± 2.3 a	7.6 ± 4.6 c	3.7 ± 3.7 ab	4.3 ± 2.7 b	<0.0001
	M. albifrons	3.3 ± 2.3 a	5.5 ± 2.6 b	3.7 ± 2.8 a	6.0 ± 2.8 b	0.0007
Total time (min) of phloem salivation (s_E1)	*A. fabae*	4.2 ± 6.6 a	8.0 ± 11.5 ab	25.9 ± 25.3 c	11.5 ± 24.1 b	0.0007
	A. pisum	4.2 ± 7.2 a	3.1 ± 4.0 a	6.4 ± 5.4 b	2.3 ± 2.0 a	0.031
	M. persicae	10.2 ± 10.8 a	10.2 ± 6.1 a	12.4 ± 14.5 a	10.0 ± 10.9 a	0.65
	A. craccivora	3.2 ± 3.5 a	9.7 ± 5.8 b	4.8 ± 4.1 a	14.0 ± 13.1 b	<0.0001
	M. albifrons	2.9 ± 2.7 a	5.2 ± 3.9 b	3.9 ± 3.3 ab	8.8 ± 14.5 b	0.0096
Time (min) from the first probe to first phloem salivation (t_1E1)	*A. fabae*	399.5 ± 278.8 a	344.8 ± 229.4 a	254.7 ± 207.4 a	328.3 ± 226.4 a	0.37
	A. pisum	501.5 ± 281.3 a	398.1 ± 268.4 a	193.7 ± 278.2 b	160.1 ± 202.9 b	<0.0001
	M. persicae	283.6 ± 228.4 a	144.8 ± 97.6 b	228.1 ± 233.7 ab	103.8 ± 79.1 b	0.015
	A. craccivora	382.5 ± 241.1 a	153.4 ± 109.9 c	303.9 ± 240.8 ab	188.3 ± 166.1 bc	0.0013
	M. albifrons	99.1 ± 58.8 a	110.1 ± 76.7 a	158.7 ± 189.6 a	38.6 ± 31.8 b	0.0002
No. complete phloem periods (n_E12)	*A. fabae*	1.7 ± 2.3 a	2.1 ± 2.3 a	2.2 ± 2.0 a	2.2 ± 3.3 a	0.48
	A. pisum	1.6 ± 2.4 a	2.8 ± 2.9 a	7.4 ± 5.8 b	2.1 ± 1.7 a	0.0002
	M. persicae	4.7 ± 4.2 ab	6.0 ± 3.7 b	3.1 ± 3.0 a	2.5 ± 2.5 a	0.0033
	A. craccivora	1.7 ± 1.5 a	5.1 ± 3.2 c	2.3 ± 2.3 ab	2.9 ± 1.7 b	0.0001
	M. albifrons	2.9 ± 1.8 ab	4.2 ± 2.6 bc	2.7 ± 1.7 a	4.4 ± 2.3 c	0.013
Total time (min) of complete phloem periods (s_E12)	*A. fabae*	12.7 ± 21.6 a	57.0 ± 56.9 b	146.5 ± 148.9 bc	259.6 ± 218.7 c	<0.0001
	A. pisum	14.0 ± 24.7 a	88.3 ± 139.9 b	234.1 ± 169.6 c	354.1 ± 188.1 d	<0.0001
	M. persicae	25.0 ± 20.1 a	234.5 ± 99.0 b	340.4 ± 196.3 c	538.3 ± 105.7 d	<0.0001
	A. craccivora	14.1 ± 19.1 a	80.0 ± 93.3 b	124.2 ± 171.8 b	185.2 ± 103.1 c	<0.0001
	M. albifrons	465.6 ± 110.8 a	405.0 ± 152.2 a	360.6 ± 217.9 a	384.5 ± 139.3 a	0.18
Time (min) from first probe to first complete phloem periods (t_1E12)	*A. fabae*	417.6 ± 273.1 a	360.0 ± 217.7 a	310.8 ± 224.7 a	346.5 ± 238.1 a	0.64
	A. pisum	501.5 ± 281.3 a	410.8 ± 268.4 a	196.2 ± 277.0 b	167.1 ± 200.1 b	<0.0001
	M. persicae	285.5 ± 227.4 a	144.8 ± 97.6 b	228.5 ± 233.4 ab	103.8 ± 79.1 b	0.013
	A. craccivora	397.1 ± 251.1 a	166.2 ± 106.1 c	359.4 ± 271.2 ab	194.3 ± 166.1 bc	0.0027
	M. albifrons	108.3 ± 68.2 a	132.6 ± 101.6 a	162.8 ± 187.1 a	42.5 ± 34.8 b	0.0004

Means within a row followed by different letters are significantly different (Kruskal-Wallis tests: $P<0.05$).

Table 3.4-6: Mean (±SD) electrical penetration graph (EPG) parameter values constituting the phloem feeding (E2) and sustained phloem feeding (sE2) of *Aphis fabae*, *Acyrthosiphon pisum*, *Myzus persicae*, *Aphis craccivora*, and *Macrosiphum albifrons* on four genotypes of *Lupinus angustifolius* during 12 h experimental time

Parameter	Aphid	Azuro	Kalya	Boregine	Bo083521AR	P
No. phloem feeding periods (n_E2)	*A. fabae*	1.7 ± 2.3 a	2.3 ± 2.6 a	2.4 ± 2.1 a	2.4 ± 3.5 a	0.39
	A. pisum	1.6 ± 2.4 a	2.9 ± 3.0 b	7.7 ± 6.0 c	2.3 ± 1.8 b	0.0001
	M. persicae	4.8 ± 4.2 ab	6.3 ± 3.9 b	3.6 ± 3.0 a	2.5 ± 2.5 a	0.0038
	A. craccivora	1.7 ± 1.5 a	5.2 ± 3.1 c	2.5 ± 2.7 ab	3.1 ± 1.8 b	<0.0001
	M. albifrons	2.9 ± 1.8 a	4.6 ± 2.6 b	2.8 ± 1.9 a	4.9 ± 2.2 b	0.0009
Total time (min) of phloem feeding (s_E2)	*A. fabae*	10.5 ± 20.6 a	52.3 ± 54.6 b	138.1 ± 146.3 bc	252.2 ± 219.2 c	<0.0001
	A. pisum	10.5 ± 21.7 a	85.7 ± 137.7 b	227.8 ± 168.3 c	352.2 ± 188.0 d	<0.0001
	M. persicae	17.9 ± 13.9 a	225.3 ± 101.1 b	328.4 ± 189.5 c	529.2 ± 108.5 d	<0.0001
	A. craccivora	12.0 ± 18.1 a	73.9 ± 92.0 b	120.7 ± 170.9 b	172.7 ± 97.1 c	<0.0001
	M. albifrons	463.5 ± 111.1 a	400.7 ± 151.9 a	357.8 ± 217.9 a	376.7 ± 133.2 a	0.14
Time (min) from first probe to first phloem feeding (t_1E2)	*A. fabae*	418.4 ± 272.4 a	361.9 ± 216.7 a	315.1 ± 223.1 a	349.4 ± 237.9 a	0.72
	A. pisum	501.9 ± 280.8 a	411.2 ± 268.1 a	196.8 ± 276.8 b	167.8 ± 200.0 b	<0.0001
	M. persicae	286.8 ± 227.1 a	146.9 ± 99.5 b	231.7 ± 231.8 ab	109.2 ± 80.7 b	0.018
	A. craccivora	398.2 ± 250.7 a	167.5 ± 106.2 c	360.7 ± 270.4 ab	195.7 ± 165.8 bc	0.0026
	M. albifrons	109.1 ± 68.2 a	133.5 ± 101.7 a	163.8 ± 187.0 a	43.3 ± 34.7 b	0.0004
No. sustained phloem feeding periods (>10 min) (n_sE2)	*A. fabae*	0.2 ± 0.4 a	0.8 ± 0.7 b	1.5 ± 1.3 b	1.6 ± 2.0 b	<0.0001
	A. pisum	0.1 ± 0.4 a	1.7 ± 2.1 b	5.6 ± 3.9 c	1.9 ± 1.6 b	<0.0001
	M. persicae	0.4 ± 0.7 a	4.1 ± 2.9 c	2.6 ± 2.8 b	2.0 ± 1.8 b	<0.0001
	A. craccivora	0.4 ± 0.7 a	1.4 ± 1.1 b	1.4 ± 1.7 b	2.2 ± 1.2 c	<0.0001
	M. albifrons	2.2 ± 1.1 a	2.8 ± 1.0 a	2.3 ± 1.6 a	4.0 ± 1.4 b	0.0002
Total time (min) of sustained phloem feeding (>10 min) (s_sE2)	*A. fabae*	5.5 ± 20.0 a	48.9 ± 54.5 b	134.9 ± 146.9 bc	250.0 ± 220.7 c	<0.0001
	A. pisum	4.0 ± 17.2 a	80.6 ± 136.5 b	216.2 ± 168.7 c	350.3 ± 188.0 d	<0.0001
	M. persicae	6.9 ± 11.7 a	214.7 ± 107.0 b	325.6 ± 189.4 c	528.0 ± 109.6 d	<0.0001
	A. craccivora	8.5 ± 15.8 a	62.9 ± 94.4 b	116.2 ± 171.8 b	169.9 ± 97.3 c	<0.0001
	M. albifrons	462.5 ± 111.4 a	397.0 ± 152.1 a	355.2 ± 220.1 a	373.8 ± 132.7 a	0.13
Time (min) from first probe to first sustained phloem feeding (>10 min) (t_1sE2)	*A. fabae*	586.6 ± 202.1 a	404.4 ± 205.9 b	342.1 ± 239.8 b	362.0 ± 236.8 b	0.0047
	A. pisum	682.0 ± 123.8 a	491.5 ± 253.4 a	202.0 ± 275.2 b	188.7 ± 220.1 b	<0.0001
	M. persicae	584.9 ± 230.1 a	169.7 ± 113.6 b	239.4 ± 228.5 b	119.3 ± 81.5 b	<0.0001
	A. craccivora	570.9 ± 252.2 a	349.1 ± 269.1 b	417.4 ± 283.7 b	230.5 ± 195.3 b	0.0025
	M. albifrons	115.5 ± 78.2 a	149.8 ± 114.0 a	175.1 ± 186.9 a	49.0 ± 51.3 b	0.0005

Means within a row followed by different letters are significantly different (Kruskal-Wallis test: $P<0.05$).

3.5. Discussion

The tested genotypes differed in the total content and composition of alkaloids and in the susceptibility for aphid feeding, indicating that the alkaloid content in the leaf tissue influences host-plant acceptance, except for the alkaloid- adapted lupin aphid *M. albifrons*. During the phase of approaching a plant and the first probing activity, aphids come into contact with surface features and plant phytochemicals (Powell et al., 1999). Schwarzkopf et al. (2013) mentioned that these factors influence the number of individuals starting penetration. Their results on the penetration behavior of the pea aphid on various legumes showed that plant volatiles and surface factors do not play an important role in host plant choice, as most aphids started probing during the EPG recording, regardless of aphid clone or plant. These results reflect the findings of the present study, in which for no genotype/aphid combination a significantly increased number of runs without recorded penetration activity was observed. Moreover, the results of studies of Caillaud (1999), describing the pea aphids' necessity for penetrating and tasting to differentiate between host and non-host plants, support our results. Further parameters that are assumed to be influenced by plant volatiles or surface factors and responsible for host plant recognition are the time from dropping the aphid on the plant surface until the first recorded probe (t_1Pr) and the duration of this first probe (d_1Pr) (Schwarzkopf et al., 2013). Results of this study showed no significant correlation between the alkaloid content and these parameters and therefore no overall repellent or attractant effect of the alkaloid content in the leaves. This indicates that the aphids are not able to recognize the alkaloid content when on the plant surface, and further surface factors or plant volatiles are assumed to be causal for significant differences between the genotypes in case of the time to the first probe observed for *M. persicae* (Table 3.4-4). Additionally, the longer time to the first probe of *A. fabae* indicates that this parameter is presumably part of a species-specific behavior.

It is stated by Tjallingii and Esch (1993) that aphids move the stylet intracellularly after penetration, puncturing cells in a more or less continuous way and sucking a small amount of cell content for gustatory analyses. It is assumed that a negative influence during this penetration period cuts down the duration of the first probe. In the present study, no significant differences between the genotypes were observed. The fact that the aphids did not abandon the first probe prematurely in the presence of a high alkaloid content leads to the conclusion that they are not able to recognize the unsuitable host during the first probing period. Zehnder et al. (2001) observed that the cowpea aphid did not perceive differences between the resistant cultivar Kalya and the susceptible cultivar

Tallerack before reaching the phloem, based on the fact that no significant differences for times of non-penetration and stylet pathway parameters were detected at the start of the recording. This is in accordance with our findings for the time from the beginning of the recording to the first probe (t_1Pr) and the duration of the first probe (d_1Pr). Annan et al. (1997) stated that for *A. craccivora*, more aphids exhibited phloem feeding on the susceptible than on the resistant cowpea cultivar, whereas the pathway parameter C did not differ between cultivars. These findings support results of this study, in which the number of aphids reaching the phloem was reduced on the high-alkaloid-containing genotype Azuro for *A. fabae*, *A. pisum*, and *A. craccivora*. As described by Pettersson et al. (2007), the final decision for host plant acceptance takes place in the phloem, although brief cell punctures occur in all tissues. The biosynthesis of quinolizidine alkaloids in Lupinus spp. takes place in the chloroplasts (Wink et al., 1982; Wink and Hartmann, 1982b) and the alkaloids are translocated via the phloem to the other plant organs, especially to the seeds, in which the highest concentration of alkaloids is present (Wink and Hartmann, 1981, 1982a; Wink and Witte, 1984). Therefore, aphids are getting into contact with alkaloids during the phloem feeding, which hints that phloem related parameters are important indicators for the acceptance of host plants. Niemeyer described that the final acceptance of a plant by an aphid is determined by the tasting of internal components, analyzed by chemosensory structures in the food canal. Therefore, it is likely that alkaloids in the phloem serve as feeding deterrents, or that they have a toxic effect on non-adapted aphids during the feeding process.

Phloem activity is recognizable beginning with a salivation period (E1) which prevents phloem wound responses, e.g., calcium-triggered coagulating proteins that can clog proteins in the phloem sieve elements (Tjallingii, 2006; Will et al., 2007). Phloem salivation always precedes phloem sap ingestion (E2) periods. In susceptible narrow-leafed lupin genotypes, an increased total time of phloem feeding in combination with a reduced stylet pathway time was observed, which is mentioned by Zehnder et al. (2001) as typical for host plant acceptance and suitability. Moreover, Montllor and Tjallingii (1989) noted that pathway pattern C occurred for longer periods when *M. persicae* probed on resistant lettuce plants, and the probes led less frequently to phloem salivation and ingestion. In contrast, the pathway duration of *M. albifrons* increased slightly with decreasing alkaloid content, which could be an indication that the lupin aphid used alkaloids as orientation cues for finding the phloem.

An indicator for non-acceptance as a host plant and therefore for aphid resistance is the reduction of phloem periods longer than 10 min, the sustained phloem feeding activity (sE2), which is a threshold time often used as a ‘phloem acceptance’ indicator (Tjallingii,

1990, 2006). Zehnder et al. (2001) observed a significantly lower proportion of phloem periods longer than 15 min of *A. craccivora* on the cultivar Kalya than on the susceptible cultivar Tallerack, and suggested that phloem in Kalya either did not stimulate feeding or contained a deterrent. The total time of sustained phloem feeding is highly correlated with the alkaloid content in our studies, indicating that alkaloid concentration prohibits sustained feeding and is therefore a major factor for the acceptance of narrow-leafed lupins as host plant of *A. fabae*, *A. pisum*, *A. craccivora*, and *M. persicae*. Consequently, resistance is believed to be located in the phloem tissue. As described by Vanhelden and Tjallingii (1993), both mechanical blocking of the sieve element after puncturing and a difference in composition of the phloem sap are possible factors for resistance. The negative correlation between phloem feeding parameters and total quinolizidine alkaloid content suggests that the composition of the phloem sap, particularly the alkaloid concentration, causes the resistance of Azuro. Although the total alkaloid content of Kalya is only 1.49 higher than that of Boregine and 5.59 higher than that of Bo083521AR, the feeding behavior of all aphid species except *M. albifrons* was negatively influenced, whereas feeding on Boregine and Bo083521AR was much more pronounced. Zehnder et al. (2001) stated that feeding deterrent compounds located in the phloem are a component of cowpea aphid resistance in the cultivar Kalya, but it must be noted that several explanations have to be taken into account. For example, a certain threshold of the total alkaloid content, a great influence of single alkaloids or their composition, the availability of other compounds of the phloem sap (e.g., amino acids), the activation of phloem sealing mechanisms triggered by stylet penetration (Caillaud and Niemeyer, 1996; Will and van Bel, A. J. E., 2006), or the absence of appropriate proteins preventing phloem sealing activities (Mutti et al., 2008) can be responsible for reduced phloem feeding. Wink (1983)conducted wounding experiments by clipping leaves with scissors, and stated that lupins have an intrinsic high level of quinolizidine alkaloids that can be amplified by wounding. However, this was not underlined by our own investigations with aphid-infested and non-infested leaves, where no significant differences in the alkaloid content were observed (Philippi, Schliephake, Jürgens and Jansen, unpubl.). In addition, investigations of Cardoza et al. (2005) showed no differences between *M. persicae* -induced and noninduced samples of narrow-leafed and yellow lupins for the levels of various defensive compounds, e.g., peroxidase, polyphenol oxidase, or proteinase inhibitors. These findings give a first indication that the varying susceptibility to aphids of lupin genotypes is most likely not due to differences in aphid-induced plant response mechanisms.

An additional influence for host and non-host reactions in EPG recordings, the so-called 'tether effect' was specified by Tjallingii (1986). He described that wiring reduces differences between host and non-host plants for penetration time and the number of penetrations. However, qualitative differences in probing behavior (e.g., occurrence of sieve element penetration) were unaffected. Wired aphids are retained on the experimental plant and forced to adjust to this situation, whereas in nature, non-wired aphids would leave the non-host plant after recognition to find a more favorable plant (Caillaud, 1999). Alvarez et al. (2013) assumed that wired aphids would probe for a longer period and more frequently than free aphids, resulting in the tendency that differences between susceptible and resistant plants are underestimated by EPG analysis. Nevertheless, a difference in probing behavior between resistant and susceptible genotypes was observed, and the results of this study show that analyzing the probing behavior of aphids by EPG technique with regard to the alkaloid content in the leaves gives a better understanding of plant resistance to aphids in narrow-leafed lupins. The results indicate that breeding for aphid-resistant narrow-leafed lupin cultivars goes hand in- hand with the alkaloid content in the leaves, leading to a challenge for breeders to meet the requirements of a reduced alkaloid content in the seeds for feed and food and coincidentally maintain a certain content and composition of alkaloids in the leaves for resistance against aphids. To solve this challenge, further aphid resistance studies should be conducted, in connection with investigations on the adequate total alkaloid content and composition of various alkaloids in the leaves.

4. Discussion

The aim of this thesis was to identify genotypes of the narrow-leafed lupin (*Lupinus angustifolius* L.) with a low total quinolizidine alkaloid content, but at the same time with resistance against five aphid species.

The common way to control aphid infestation in the absence of resistant cultivars is the application of insecticides. Guidance for lupin cultivation in Australia indicates varieties susceptible to aphid infestation for which spraying for control can be vital (Garlinge, 2005). It was reported that insecticides containing e.g. the active substance alpha-cypermethrin are effective against *M. albifrons* and *A. craccivora* or the active substance imidacloprid for control of *M. persicae* (Thackray et al., 2000; Dewar, 2007). However, the use of pesticides is more and more restricted due to potential risks for humans, animals and the environment. For example, the use of imidacloprid was strongly restricted in 2018 in the European Union as laid down in Commission Implementing Regulation (EU) 2018/783 (European Union, 2018), saying that plant protection products containing imidacloprid are only to be used in permanent greenhouses and the treated crops must be kept within a permanent greenhouse during its entire life cycle, which is mainly based on the high risk of harm to honey bees and other pollinators. Moreover, the Sustainable Use Directive (European Union, 2009) and the National Action Plans (NAP) of the EU member states established the target for reduction of the use of pesticides released to the environment. It also has to be mentioned that the demand for organic production of food increases steadily, and possibilities for aphid control in organic farming are very limited. Taking into account the predicted climate change, with rising temperatures and prolonged growing seasons, the generation number of aphids is expected to increase (Yamamura and Kiritani, 1998; Hullé et al., 2010). This facilitates on the one hand a faster development of resistance against insecticides for multivoltine insects like aphids and on the other hand the adaptation to new hosts and a permanent establishment as a pest, besides the enhanced risk for yield losses due to aphid feeding directly and indirectly by virus transmission (Foster et al., 2007; Loxdale and Lushai, 2007; Tobin et al., 2008). All these aspects justify the need for breeding of aphid-resistance in narrow-leafed lupin.

Screening trials for aphid multiplication potential

In total 46 genotypes were examined in screening trials with regard to their potential for multiplication of *Aphis fabae, Aphis craccivora, Acyrthosiphon pisum, Myzus persicae* and *Macrosiphum albifrons* on these genotypes and their QA composition, with the aim to identify resistant genotypes likewise with a low QA content.

It was reported by French (2004) that there are differences between Australian narrow-leafed lupin cultivars with regard to their susceptibility for aphid feeding and its resulting damage, which is supported by results of Berlandier and Sweetingham (2003), showing cultivar-dependent differences in yield losses caused by infestation with *A. craccivora, M. persicae* and *Apcyrthosiphon kondoi* in Australian field trials. Cultivar-dependent differences in aphid multiplication were also observed in our investigations. As expected, a direct influence of the total QA content was shown. An exception was shown for the well-adapted lupin aphid (*M. albifrons*), for which no suppressing influence of the QA content was detected. This coincides with previous findings, where an infestation with *M. albifrons* was only influenced to a minor extent by differences between *Lupinus albus* cultivars (Ferguson, 1994).

Leaving *M. albifrons* aside, the highest multiplication rates on genotypes with a low QA content were generally observed for *A. fabae*, while no multiplication of *A. fabae* on genotypes with a high QA content was observed. This is in line with findings of Gruppe and Roemer (1988) and Ferguson (1994), showing that *A. fabae* can be observed on sweet lupins, but is not able to colonize lupins with a high QA content. For *M. persicae* similar results were shown, with a slightly lower multiplication potential on sweet genotypes compared with *A. fabae* but the same incapability to colonize genotypes with a high QA level. These findings coincide with previous reports, that the potential of *M. persicae* to infest narrow-leafed lupins with a low alkaloid content is high, but development on QA-rich genotypes is suppressed (Wink, 1992; Berlandier, 1996; Edwards, 2001).

Multiplication of *A. pisum* and *A. craccivora* was only observed on a low number of the genotypes investigated, all of them with a low total QA content. However, it is well-known in particular from Australia, that *A. craccivora* is generally able to colonize susceptible cultivars and is capable to cause severe yield losses (Zehnder et al., 2001; Berlandier and Sweetingham, 2003). Edwards et al. (2003) investigated three *L. angustifolius* cultivars (‘Tallerack’, ‘Tanjil’ and ‘Kalya’), and concluded that *M. persicae* can tolerate alkaloids (or other allelochemicals) to a higher extent than *A. craccivora* due to a higher growth and survival rate, which coincides with our findings. However, it was reported by

Edwards et al. (2003) in field trials after artificial infestation that a higher number of *A. craccivora* was observed on the susceptible cultivar 'Tallerack' then of *M. persicae*, which was the opposite on cvs. 'Tanjil' and 'Kalya' as moderate resistant to resistant cultivars, respectively.
Furthermore, findings of several authors showed the susceptibility of genotypes with a low QA content for *A. pisum* colonization, which is incapable to infest QA-rich genotypes (Gruppe and Roemer, 1988; Kordan et al., 2008; Kordan et al., 2012).

In general, aphids are able to exploit new host species and overcome novel resistance mechanisms in newly developed plant varieties due to their phenotypic plasticity (Cardoza et al., 2006). Such adaptation mechanism is described by Cardoza et al. (2006) for *M. persicae,* showing the superiority of one clone collected from Western Australia (extensive lupin cultivation) compared with nine clones from Eastern Australia (scarce lupin cultivation). Thus, continuous cultivation of genotypes with a QA content as low as possible provides the potential for host-plant adaptation, and may lead to higher infestation rates and a more widespread set of genotypes which can be infested e.g. by *A. pisum* or *A. craccivora*, respectively. As shown in the screening trials the breeding line 'Bo083521AR' with the lowest QA content was most susceptible to aphid multiplication of all species investigated (with exception of *M. albifrons*), and it can be assumed that permanent cultivation of genotypes with such susceptibility level would lead to an enhanced adaptation on narrow-leafed lupins as host plant.

Within the screening trials three genotypes were identified to be resistant against aphids (except *M. albifrons*), namely 'Borlu', 'Bora' and 'Kalya', the latter already identified in previous investigations as a resistant cultivar with a low QA-content (Zehnder et al., 2001; Berlandier and Sweetingham, 2003; Edwards et al., 2003; Adhikari et al., 2012). Due to the fact that these genotypes showed aphid resistance although having a low total QA content, a correlation between aphid multiplication and QA composition besides total QA content was assumed.

Results of the QA analysis showed, that generally 13-hydroxylupanine was prevalent with the largest share in the total QA content in the narrow-leafed lupin genotypes investigated, followed by angustifoline and 13-tigloyloxylupanine. Furthermore, the QAs lupanine, 13-trans-cinnamoyloxylupanine, 13-cis-cinnamoyloxylupanine, 13-benzoyloxylupanine, isolupanine and tetrahydrorhombifoline were detected in all genotypes investigated, while multiflorine and sparteine were only found in some genotypes and in small amounts or even traces.

Reactions of aphids to specific QAs prevalent in lupins have already been described in the past. Berlandier (1996) concluded that the alkaloid level may suppress the fecundity of *M. persicae.* Furthermore, it was reported, that lupins (e.g. *L. luteus*) are avoided by *M. albifrons* when containing the bicyclic QA lupinine (Wink and Roemer, 1986; Gruppe and Roemer, 1988), which is not present in the narrow-leafed lupin. These findings substantiate the result of this thesis, showing the varying influence of different QAs, and that particular QAs can have a high activity and high influence on aphids and their host plant acceptance, independently from the total QA content.

Findings of Ridsdill-Smith et al. (2004) showed in alkaloid feeding bioassays that *M. persicae* is less affected by feeding on diets containing alkaloids than *A. craccivora*, which is in line with the results of our screening trials. They showed that 13-hydroxylupanine and angustifoline had only little to no effect on *M. persicae*, while for *A. craccivora* a significant effect on aphid survival and growth was observed. Correlation analysis for *M. persicae* and *A. craccivora* in screening trials also showed a substantial negative correlation between 13-hydroxylupanine content and the multiplication (average ordinate of population growth) of *A. craccivora* (Spearman's correlation coefficient r= -0.526). For *M.* persicae (r= -0.425) also a significant negative correlation was shown, which is in contrast to the findings of Ridsdill-Smith et al. (2004). However, their results for angustifoline were verified in our findings, showing a negative effect on *A. craccivora* multiplication (r= -0.433 significant) but no or only a very limited effect on *M. persicae* (r= -0.194; non-significant).

Ridsdill-Smith et al. (2004) also reported, that lupanine is the only QA having a larger effect on *M. persicae* than on *A. craccivora*. In our findings, only a low negative correlation between the lupanine content and the multiplication of *M. persicae* (r= -0.276; significant) and *A. craccivora* (r= -0.153; non-significant) was observed, but also with a slightly higher influence on *M. persicae.*

Dreyer et al. (1985) showed in diet experiments that (among others) the lupin QA L-sparteine inhibits feeding of the pea aphid, *Acyrthosiphon pisum.* In our investigations, sparteine was also detected but in contrast to the results of Dreyer et al. (1985) a weak positive correlation between the sparteine content and *A. pisum* (r=-0.284; significant) multiplication was shown, with similar findings for all other aphids except *M. albifrons.* This might be due to the relatively low amount of sparteine detected in the genotypes analysed.

Results of multiple regressions between aphid multiplication and QA content and composition of 33 narrow-leafed lupin genotypes with a QA total content <1000 µg/g dry leaf matter (identified as threshold for aphid multiplication) showed that especially the

QA 13-tigloyloxylupanine influences the multiplication of *A. fabae*, *A. pisum*, *A. craccivora* and *M. persicae* on narrow-leafed lupins.
The pathway of 13-tigloyloxylupanine biosynthesis starts with the amino acid lysine, out of which cadaverine is derived by decarboxylation, which is the precursor of lupanine (among others) (Hirai et al., 2000). Lupanine is transformed by enzymatic reaction to 13-hydroxylupanine, out of which 13-tigloyloxylupanine is built by esterification initiated by the enzyme I3α-hydroxylupanine-0-tigloyltransferase (Suzuki et al., 1994; Okada et al., 2005). Hirai et al. (2000) and Saito et al. (1993) found the same amount of I3α-hydroxylupanine-0-tigloyltransferase in both, sweet and QA-rich *L. angustifolius* genotypes, and due to the fact, that lysine and cadaverine were found in the same amount they postulated that the enzymatic reaction from cadaverine to lupinin and other cyclic alkaloids is the limiting step in genotypes with a low QA content. Based on these results and the biosynthesis pathway it may be concluded that the alkaloid synthesis should not be completely down regulated, to preserve a sufficient 13-tigloyloxylupanine content leading to resistance against aphids. The analysis of the content of 13-tigloyloxylupanine may be used as an indirect selection criterion in breeding, and may be used as the starting point for investigating the genetic basis of aphid resistance as found in the genotypes ‘Kalya’, ‘Bora’ and ‘Borlu’.

Feeding behaviour of aphids on narrow-leafed lupins - EPG

The feeding behaviour of *A. fabae, A. craccivora, A. pisum, M. persicae* and *M. albifrons* was investigated on four narrow-leafed lupin genotypes, selected due to differences regarding the aphid multiplication potential in the screening trials, and thus the direct influence of the QA content on the aphid species was examined. Investigations to identify differences of feeding behaviour were conducted on the genotype with the lowest total QA content (‘Bo083521AR’), ‘Boregine’ as cultivar with a low QA content but reduced aphid multiplication, the cultivar ‘Kalya’ with low QA content but aphid resistance and the cultivar ‘Azuro’ with a high QA content.
In addition to the cultivar ‘Kalya’, which was already identified in previous investigations as a resistant cultivar with a low QA-content (Zehnder et al., 2001; Berlandier and Sweetingham, 2003; Edwards et al., 2003; Adhikari et al., 2012) also other promising genotypes with a low QA content and aphid resistance (except for *M. albifrons*) were detected in the screening trials (i.e. ‘Borlu’ and ‘Bora’). However, due to the already reported findings of Zehnder et al. (2001), where *A. craccivora* showed a reduced phase of sieve element feeding on the cultivar ‘Kalya’ compared with a susceptible cultivar,

'Kalya' was also used for our investigations of feeding behaviour of several aphid species.

The investigation of quinolizidine alkaloids in (narrow-leafed) lupins has a long history, based especially on the extensive work of Wink (1985, 1987a, 1988, 1992, 2011). QAs are derived from the amino acid lysine (Frick et al. (2017)) and it was shown by Wink and Hartmann (1982b) that, as known for lysine formation, the biosynthesis of quinolizidine alkaloids takes place in the chloroplast, and the QAs are then exported from the leaves to other plant organs. In stems and leaves of *Lupinus* spp. alkaloids are predominantly sequestered in epidermal cells (Wink and Hartmann, 1982b; Wink, 1986). Wink (1992) showed that the composition of quinolizidine alkaloids of a plant is most complex in the leaf, but is usually somewhat different in other organs, such as seeds. This is possibly reflecting selective transport and/or metabolic transformations. Translocation of quinolizidine alkaloids from the chloroplast all over the plant takes place by the phloem and not the xylem (Wink and Hartmann, 1982a; Wink and Witte, 1984, 1991; Wink, 1992).
Taking into account the biosynthesis of alkaloids and the localisation within plant tissues, it was postulated that an influence of the QA content on the feeding behaviour as such and in particular on specific probing phases can be detected by EPG measuring.

No significant differences were observed in the number of runs concerning probing activity between the aphid-genotype combinations. Aphids are coming into contact with surface features and plant phytochemicals during settlement on a potential host plant, which can influence the number of aphid individuals starting with probing (Powell et al., 1999; Schwarzkopf et al., 2013). Due to the fact that for the number of runs with probing activity no differences between the genotypes were observed it can be concluded that plant volatiles and surface factors of narrow-leafed lupins have no important role in host plant choice of aphids, and thus for differentiation between host and non-host plants penetration and tasting of plant tissue is necessary.

During aphid penetration, two distinct phases are described. On the one hand the pathway phase, comprising extracellular stylet penetration crossed by brief cell punctures in non-vascular tissues. This is linked to ingestion of cell contents that may be examined by chemoreceptors in the epipharyngeal organ, and thus such sap sampling from non-phloem tissue may serve for gustatory analysis (Tjallingii and Esch, 1993; Gabrys and Tjallingii, 2002; Dancewicz et al., 2016). On the other hand the phase of ingestion is characterized by intake of sap from vascular tissues (Dancewicz et al., 2016). The latter phase contains (i) the ingestion of sap from phloem sieve tubes, preceded by

a salivation period which prevents phloem wound responses such as calcium-triggered coagulating proteins that can clog proteins in the phloem sieve elements, and (ii) the ingestion from xylem vessel elements (Tjallingii, 2006; Pettersson et al., 2007; Will et al., 2007).

For the time to the first probe (pathway phase) as well as the duration of the first probe, which are both parameters related to host plant recognition (Schwarzkopf et al., 2013), no influence of the total QA content of the investigated *L. angustifolius* genotypes was observed. Although brief cell punctures in non-vascular tissues are conducted during the pathway phase, no significant differences related to the total QA content were observed at this early time of probing. Thus, it can be assumed that during aphids gustatory analysis by cell puncturing as described by Tjallingii and Esch (1993), either a high QA content is not detected, or does not function as a direct repellent at this early stage of acquisition, or due to the fact that the main translocation route of QAs is located in the phloem (Wink, 1992) an acceptable level of QAs prevails in epidermis and parenchyma cells of high-alkaloid cultivars. Findings of Zehnder et al. (2001) substantiate these results, as no differences were observed between probing behaviour on 'Kalya' and the susceptible cultivar 'Tallerack' when investigating the times of non-penetration and stylet pathway parameters of *A. craccivora* at the beginning of recording.
For *A. fabae* a longer time to the first probe compared with all other aphid species was detected on all genotypes investigated, which indicates that *A. fabae* has a species-specific longer period of host plant approaching on narrow-leafed lupins.

In summary, it can be concluded that the aphids are unable to recognize the unsuitable host containing a high QA content at an early stage of the first probing period. This is in line with the conclusion of Pettersson et al. (2007), that the final decision for host plant acceptance takes place in the sieve elements. Most significant influences of the QA content were indeed observed for phloem related parameters for all aphids except *M. albiforns*, which is linked to the fact that the main route of QAs translocation is via the phloem (Wink and Hartmann, 1982a; Wink and Witte, 1984, 1991; Wink, 1992). Since the phloem is a target for many sucking insects, such as aphids, these insects usually avoid plants with a high load of alkaloids in the phloem (Wink and Mohamed, 2003) and thus a high QA concentration in the phloem sap leads to deterring effects for non-adapted aphid species. In susceptible genotypes, an increase of the total time of phloem feeding was observed together with a reduced stylet pathway time. Such findings were also reported by Montllor and Tjallingii (1989), investigating the feeding behaviour of *M. persicae* probing on susceptible and resistant lettuce plants, and Zehnder et al. (2001) identified this as a typical behaviour showing host plant acceptance and suitability.

Therefore, it can be assumed that quinolizidine alkaloids in the phloem serve either as feeding deterrents or have a toxic effect during feeding on non-adapted aphid species.
For *M. albifrons* a slightly increased pathway duration was observed in low-QA genotypes. Gruppe and Roemer (1988) reported, the QAs serve the lupin aphid as a cue to find suitable host plants, thus it can be concluded from our results that a reduced QA content facilitates phloem approaching for *M. albifrons.*
A major indicator for host-plant acceptance or denial is the sustained phloem feeding for more than 10 min, which is according to Tjallingii (1990) a threshold time indicating phloem acceptance of the aphid. Results showed a clear negative correlation between the sustained phloem feeding and the QA content, thus sustained feeding of *A. fabae, A. pisum, A. craccivora*, and *M. persicae* is inhibited by a high QA concentration in the phloem and can be regarded as an important factor for host plant acceptance of narrow-leafed lupin genotypes.

When speaking about EPG analysis the so-called 'tether effect' has to be taken into account, which comprises effects on the wired aphid which are forced to adjust to the situation and to stay on the experimental plant, whereas under natural conditions aphids would leave the non-host plant to move to a more favorable plant. It is assumed that tethered aphids tend to probe for a longer period and more frequently which obliterates differences between susceptible and resistant plants (Tjallingii, 1986; Caillaud, 1999; Alvarez et al., 2006). Although an influence of the so-called 'tether effect' cannot be completely excluded, differences in the probing behaviour between susceptible and resistant genotypes of the narrow-leafed lupin were observed. Results of the investigation of probing behaviour of different aphid species in correlation with the QA content in the leaves facilitate the understanding of resistance of narrow-leafed lupins to aphids.

It can generally be concluded, that based on the results for the first probing the pathway parameters and in particular the phloem related parameters, the resistance of narrow-leafed lupins is located in the sieve element and is highly correlated with the QA content. However, especially with regard to the findings on cultivar 'Kalya' with aphid resistance in coincidence with a low QA total content, several possibilities for phloem related resistance have to be considered. Not only the total content, but in particular the composition of different QAs in the phloem sap was shown from screening trials to influence aphid development. For example, 13-tigloyloxylupanine, detected in the screening trials as an important factor for aphid resistance, is under suspicion to have a severe effect on the feeding behaviour as such and the phloem feeding in particular.

Another option is the upregulation of QAs (in general or of specific QAs) due to wounding of plant tissues by aphid feeding. It was reported from wounding experiments that the amount of quinolizidine alkaloids can be amplified by wounding (Wink, 1983). This was not proved in our own investigations with aphid-infested and non-infested leaves, showing no significant differences in the QA content (unpublished data). Frick et al. (2019) investigated, whether aphid infestation may increase the level of QAs in grains of two cultivars of narrow-leafed lupins, one sweet and one with a high QA content. However, an influence of aphid feeding on the QA content was not verifiable, and the authors concluded that aphids are not a concern for increasing grain QAs in narrow-leafed lupin cultivars. This is in line with investigations of Cardoza et al. (2005), in which no differences between *M. persicae* infested and control samples of narrow-leafed and yellow lupins for the levels of various defensive compounds (e.g., peroxidase, polyphenol oxidase, or proteinase inhibitors) were detected. Therefore, an aphid-induced plant response mechanism as basis for varying susceptibility to aphids of different lupin genotypes is unlikely.

In addition, the QA content often varies due to environmental conditions (Cowling and Tarr, 2004). To decrease the influence of environmental factors and to contribute to the observation of Wink and Hartmann (1982a) showing a diurnal fluctuation of quinolizidine alkaloid accumulation in *Lupinus polyphyllus* with an increase of the alkaloid contents in the light and a decrease during the dark, our trials were conducted under controlled conditions. Thus, the influence of environmental factors on the QA content was reduced to the minimum.

Moreover, other factors such as the bioavailability of additional compounds like amino acids in the phloem sap may influence the feeding behaviour, or the activation of phloem sealing mechanisms as a response to stylet penetration as well as the absence of suitable proteins for the inhibition of phloem sealing activities may be responsible for phloem related resistance (Caillaud and Niemeyer, 1996; Will and van Bel, A. J. E., 2006).

However, results of all investigations from screening trials as well as the observations of the feeding behaviour revealed a strong negative correlation between aphid development and QA content and composition in the phloem, respectively. Therefore, breeding for QA related aphid resistance seems to be promising.

Outlook for future breeding

A key for breeding efforts is the availability of genetic variability, and in this respect the presence of a genetically diverse set of genotypes. According to Gresta et al. (2017) for *L. angustifolius* 3894 accessions are available in gene banks worldwide.

As described in the chapters above, genetic diversity regarding the resistance or reduced susceptibility of *L. angustifolius* for feeding of non-specialised aphid species was shown, even in a comparatively reduced set of genotypes with 46 accessions and varieties. The results from these aphid resistance screening tests as well as the results from EPG recordings showed linkage between specific QAs and the multiplication and the feeding behaviour of *A. fabae, A. craccivora, A. pisum* and *M. persicae*, which might be used as starting point for searching the underlying genetic basis of resistance against common aphid species. It was found that 13-tigloyloxylupanine can play an important role in aphid resistance, so that breeding of genotypes with a low total QA content but a relatively high 13-tigloyloxylupanine content may be a good option for breeding narrow-leafed lupins suited for food or feed and being aphid resistant. The content of 13-tigloyloxylupanine may be used as an indirect selection criterion, to prevent the challenging task of including aphid rearing and determination of the multiplication rate into practical lupin breeding.

Classical breeding is more and more displaced by breeding based on marker-assisted selection. Extensive work with regard to sequencing of the genome of *L. angustifolius* was conducted within the past years. Genetic maps of the narrow-leafed lupin have been developed and extensive work with regard to establishment of molecular markers was performed (Boersma et al., 2005; Nelson et al., 2006; Gao et al., 2011; Yang et al., 2013c; Wyrwa et al., 2016; Hane et al., 2017). A comprehensive review of available genomic tools in *L. angustifolius* and *L. albus* is published in Abraham et al. (2019).

Among others, the narrow-leafed lupin genome project (Singh and Kamphuis, 2019) was established, and in this context the comprehensive draft genome sequence of *L. angustifolius* was published (Hane et al., 2017). With this, a platform for genome-wide association studies and genomics-based breeding programs is provided. Breeding for major agronomic and physiological traits in narrow-leafed lupins was already facilitated by the development of molecular markers e.g. for anthracnose resistance (Yang et al., 2004; You et al., 2005; Yang et al., 2013a; Fischer et al., 2015; Hane et al., 2017). Furthermore, molecular markers were developed referring to Phomopsis stem blight by next-generation sequencing (Yang et al., 2013b), and to the early-flowering gene *Ku,* which removes the requirement for vernalization (Boersma et al., 2007).

Starting point for analyses of the genetic background for aphid resistance related to QAs in narrow-leafed lupins may be based on findings of Okada et al. (2005) for *Lupinus albus*, where the gene encoding for tigloyl-CoA:(–)-13α-hydroxymultiflorine/(+)-13α-hydroxylupanine O-tigloyltransferase (HMT/HLT) was published, which catalyses (among others) the formation of (+)-13α-tigloyloxylupanine from (+)-13α-hydroxylupanine. As described above, 13-tigloyloxylupanine was identified to have an important impact on aphid multiplication and thus investigations of the genetic background for 13-tigloyloxylupanine biosynthesis may give first hints of the mechanism underlying the aphid resistance of narrow-leafed lupins.

In addition genes for lysine decarboxylase (LDC) and cadaverine oxidase have been identified, and according to Wink (2018) analyses of transcriptomes from QA producing plants, generated by RNASeq, to identify additional genes involved in QA synthesis, storage and transport is currently ongoing, which may then be used for further investigations.

Another approach for breeding of aphid resistant narrow-leafed lupins with a low QA content in the seeds is described by Wink (2011) and Wink (2018). It is postulated that the transfer from the phloem into the growing seeds requires an alkaloid transporter, which was identified by Frick et al. (2017). A knock out of this transporter may facilitate the creation of lupins with high QA levels in the green parts but low levels in the seeds. As a consequence, such lupins would maintain resistance against herbivores but would produce seeds with a low, palatable content of quinolizidine alkaloids.

Taking the above approaches into account, breeding for aphid resistance of narrow-leafed lupins in coincidence with a low quinolizidine alkaloid content in seeds can be regarded as encouraging. This joins the requirements for its use as food and feed with the requisite of improved yield stability.

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List of publications and conference contributions

Publications in peer-reviewed journals with respect to this thesis:

Philippi J, Schliephake E, Jürgens H-U, Jansen G, Ordon F, 2015. Feeding behavior of aphids on narrow-leafed lupin (*Lupinus angustifolius*) genotypes varying in the content of quinolizidine alkaloids. Entomol Exp Appl 156, 37–51. DOI: 10.1111/eea.12313.

Philippi J, Schliephake E, Jürgens H-U, Jansen G, Ordon F, 2016. Correlation of the alkaloid content and composition of narrow-leafed lupins (*Lupinus angustifolius* L.) to aphid susceptibility. Journal of Pest Science 89, 359–373. DOI: 10.1007/s10340-015-0710-y.

Conference contributions with repect to this thesis (and publ. in abstract journals):

Philippi, J. (Poster); Schliephake, E.; Ordon, F. (2012): Penetration behavior of different aphid species on *Lupinus angustifolius* L. genotypes. In: JKI (Hrsg.): Fünftes Nachwuchswissenschaftlerforum 2012, 4. - 6. Dezember in Quedlinburg ; Abstracts (Berichte aus dem Julius Kühn-Institut 167), Ribbesbüttel, 45.

Philippi, J. (Oral); Schliephake, E.; Ordon, F. (2012): Selektion von Lupinen-Genotypen mir Resistenz gegen Aphiden und deren Abhängigkeit von Alkaloidgehalt und Entwicklungstemperatur.
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Philippi, J. (Oral); Jansen, G.; Jürgens, H.-U.; Kaufmann, K.; Seddig, S; Schliephake, E. (2013): Die Blaue Süßlupine - Züchtung im Spannungsfeld von Alkaloidgehalt und Blattlausanfälligkeit. Senatsarbeitsgruppe „Ökologischer Landbau“, Dummerstorf. Published as: Philippi et al. 2013; Blattläusen das Leben schwer machen, die Blaue Lupine als Eiweißpflanze weiterentwickeln. In: Forschungsreport Spezial Ökologischer Landbau, No. 2, pp. 14-15

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8. Eidesstattliche Erklärung / *Declaration under Oath*

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

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