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EARLY LAND PLANTS: PLENTIFUL BUT NEGLECTED RESOURCES FOR HERBIVORES?

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Author: Audrey DUHIN

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Supervised by: Pr. Gregory Roeder

Co-supervised by: Pr. Ted C. J. Turlings

Committee members: Pr. Gregory Roeder
Pr. Ted C. J. Turlings
Pr. Sergio Rasmann
Pr. Laure Weisskopf

Université de Neuchâtel
Faculté des sciences

Rue Emile-Argand 11
CH-2000 Neuchâtel
doctorat.sciences@unine.ch

IMPRIMATUR POUR THESE DE DOCTORAT

La Faculté des sciences de l'Université de Neuchâtel autorise l'impression de la présente thèse soutenue par

Madame Audrey DUHIN

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sur le rapport des membres du jury composé comme suit:

- Prof. tit. Gregory Roeder, directeur de thèse, Université de Neuchâtel, Suisse
- Prof. Sergio Rasmann, Université de Neuchâtel, Suisse
- Prof. Théodoor Turlings, Université de Neuchâtel, Suisse
- Prof. Laure Weisskopf, Université de Fribourg, Suisse

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Le Doyen, Prof. R. Bshary

Résumé

Il existe entre les organismes vivants de nombreux types d'interactions. Parmi ceux-ci, figure l'herbivorie qui est la plupart du temps un cas d'antagonisme, au même titre que les relations proie-prédateur. Il s'agit d'un phénomène qui est assez bien étudié pour certains groupes de plantes comme les angiospermes (plantes à fleurs), dont le niveau d'évolution est considéré comme le plus élevé, d'où leur nom de « plantes supérieures ». À contrario, d'autres types de plantes sont moins étudiées de manière générale, et en particulier au sujet de l'herbivorie. C'est le cas des mousses, qui sont les premières espèces végétales à avoir colonisé l'habitat terrestre. Celles-ci ont par conséquent dû développer diverses stratégies lors de leur sortie de l'environnement aquatique, comme une résistance aux rayons UV ainsi qu'à la dessiccation. Les fougères sont également sous-étudiées alors qu'elles sont quant à elles, les premières à présenter un système vasculaire, nécessaire à une croissance supérieure à une dizaine de centimètres à laquelle sont contraintes les mousses.

Les plantes sont constamment soumises à différents stress dans leurs milieux. Ceux-ci peuvent être abiotiques (*i.e.* dus à des facteurs environnementaux non vivants tels que la température, l'humidité, la nature du sol, etc.) ou biotiques (*i.e.* dus à des organismes vivants tels que des pathogènes, des herbivores, d'autres plantes en compétition, etc.). Dans ce travail, une attention particulière est portée sur l'herbivorie. Dans ce cas précis, les végétaux doivent adopter une stratégie pour survivre et plusieurs possibilités s'offrent à eux comme de tolérer les attaques ou de se défendre. L'option de défense se décline en plusieurs facettes, comme de contenir peu de nutriments ou d'arborer des structures physiques comme des épines ou des trichomes. Une autre possibilité est de contenir des molécules chimiques toxiques, pouvant être volatiles ou non. Ces défenses peuvent être combinées et sont également parfois inductibles suite à un stress particulier, phénomène qui est régulé par des phytohormones au sein de la plante.

Dans ce travail de thèse, différents types de défenses sont étudiés chez les plantes primitives que sont les mousses et les fougères. Dans un premier chapitre, les recherches se sont focalisées sur le volet nutritif des plantes terrestres primitives. Ainsi, le contenu en nutriments de 15 mousses et fougères a été analysé en termes de protéines, acides aminés et sucres. En parallèle, des feuilles ont été données à manger à deux types d'herbivores : des escargots (*Cochlicella baraba*) et des chenilles (*Spodoptera exigua*). Ces deux espèces phytophages généralistes ne se sont pas bien portées ni développées sur les traitements proposés, à l'exception de la diète artificielle et du maïs, utilisés comme contrôles. Cependant, il a été démontré par ce biais que ces végétaux ont des niveaux nutritifs suffisamment élevés pour permettre le développement des organismes phytophages, mais que les compositions en acides aminés et en sucres différents qualitativement.

Dans un second chapitre, ce sont les défenses et facteurs physiques qui ont été abordés. Cette fois encore, des bioessais avec des chenilles ont été effectués, mais à l'aide de diète artificielle agrémentée de poudre de plantes primitives. Les insectes utilisés pour cette expérience se sont bien développés sur les diètes contenant du tissu provenant d'une des espèces de mousse, de fougère aquatique ou des plantes à fleurs utilisées comme contrôle. Les performances ont par contre été à nouveau mauvaises sur les espèces de fougères terrestres. Par ailleurs, les éventuelles structures défensives telles que des trichomes ont été investiguées et seule l'espèce de fougère aquatique incluse dans l'étude en présentait à des fins de flottaison. Des analyses du contenu en silice ont été également effectuées et il est assez variable au sein d'une même espèce étudiée et il n'a pas été possible de détecter une différence significative entre les différentes plantes. De plus, aucune corrélation entre le contenu en cet élément n'a pu être établie. La concentration en eau complète les données de cette partie et cette fois, il a été possible de différencier de manière statistiquement significative les espèces de fougères terrestres présentant un contenu en eau inférieur au reste des végétaux employés. Cela suggère cependant que ces défenses physiques ne sont pas impliquées dans la stratégie des plantes inférieures pour lutter contre les herbivores.

La suite logique exploitée dans un troisième volet sont les défenses chimiques. Pour cette partie, seules deux espèces de ptéridophytes ont été utilisées, à savoir un lycopode et une fougère. Pour mener à bien les investigations de ce volet, des extractions de composés chimiques ont été réalisées à l'aide de différents solvants tels que de l'eau combinée à 0,1% d'acide formique, du méthanol et du dichlorométhane. Les extraits ainsi obtenus ont été séchés et mélangés à de la diète artificielle afin de nourrir des chenilles polyphages. Celles-ci ont éprouvé des difficultés à gagner du poids et à se développer sur deux des extraits effectués sur la fougère. Celui réalisé avec un solvant aqueux a été fractionné et ces parties à nouveau ajoutées à de la diète artificielle pour être également proposée à des larves de mites. L'une des fractions s'est avérée plus toxique que les autres et contenait des phloroglucinols, une classe de molécules connue pour être impliquée dans la défense de plantes comme l'eucalyptus. Il a donc été conclu que ces molécules jouent probablement le même rôle pour l'espèce de fougère étudiée ici.

Dans un quatrième chapitre de ce travail, ce sont les phytohormones et les composés organiques volatils (COVs) émis par une espèce de fougère qui ont été investigués. Ces composés ont été mesurés après l'attaque de pucerons spécialisés (*Idiopterus nephrelepidis*) ou de chenilles (*S. exigua*), ou encore suit à des dégâts mécaniques. Il n'a pas été possible de détecter une réponse hormonale des plantes, ni de détecter de changement au niveau des COVs émis suite à l'agression par les insectes piqueurs-suceurs. En revanche, il a été démontré que cette espèce de fougère change d'odeur lorsqu'elle est consommée par la chenille ou soumise à des blessures seules, ces deux cas étant discernables entre eux. Ce mécanisme n'est donc pas limité aux plantes à fleurs et pourrait être utile aux ptéridophytes pour, par exemple, attirer des auxiliaires comme des parasitoïdes, les aidant à lutter contre les assaillants.

Dans l'ensemble, les résultats de ce travail de doctorat démontrent que la problématique de l'herbivorie chez les plantes inférieure est complexe et mérite l'attention de la recherche, tout en soulignant l'importance de l'étude de ces groupes de plantes dans une perspective évolutionnaire. De plus, ces plantes sont dites primitives, mais nous observons les représentants actuels qui font face aux mêmes contraintes que les plantes dites supérieures et ont dû s'adapter tout autant qu'elles à leur environnement.

Mots-clés : Plantes terrestres primitives, Herbivorie, Interactions plante-insecte, Bryophytes, Ptéridophytes

Summary

There are many types of interactions between living organisms. Among these, we can find the herbivory interaction. This is most of the time a case of antagonism, as are prey-predator relationships. This is a phenomenon that is fairly well studied for certain groups of plants such as angiosperms (flowering plants). These plants are considered to be at the highest level of evolution and are referred as "higher plants". On the other hand, other types of plants are less studied in general, and in particular on the subject of herbivory. This is the case for the bryophytes, which are the first plant species to have colonized the terrestrial habitat. They had to develop various strategies during their ground colonization, such as resistance to UV radiation and desiccation. Ferns are also under-studied, but they are the first group in evolution presenting a vascular system, necessary for a growth superior to about ten centimeters to which mosses are constrained.

Plants are constantly subjected to different stresses in their environments. These can be abiotic (*i.e.* due to non-living environmental factors such as temperature, humidity, soil type, etc.) or biotic (*i.e.* due to living organisms such as pathogens, herbivores, other plants in competition, etc.). In this work, a particular focus is made on herbivory. In this interaction, plants must adopt a strategy to survive to herbivores’ consumption and several possibilities are offered to them. Indeed, they can tolerate the attacks or defend themselves. This last option is declined in several facets, like having a low nutrients content or like exhibit physical structures as spines or trichomes. Another possibility is to contain toxic chemical compounds, which can be volatile or not. These defenses can be combined and are also sometimes inducible by a particular stress. This phenomenon is regulated by phytohormones in the plant.

In this thesis, different types of defenses are studied in primitive plants such as mosses and ferns. Thus, in a first chapter, the nutrient content of 15 mosses and ferns was analyzed in terms of proteins, amino acids and sugars. In parallel, leaves were fed to two types of herbivores: snails (*Cochlicella baraba*) and caterpillars (*Spodoptera exigua*). These two generalist phytophagous species do not survive nor develop sustainably to their reproductive adult stage on the proposed treatments, except for the artificial diet and maize, used as controls. However, it was shown that these plants have a high enough nutrient level to allow development, but that the compositions in amino acids and sugars differ qualitatively.

In a second chapter, defenses and physical factors were addressed. Bioassays with caterpillars were also carried out in this part. The treatment proposed to the herbivores were artificial diet mixed with powder made from 15 different primitive plants and two flower plants or no plant used as control. We obtained results showing that the insects grew well on diets containing tissue from one of the bryophyte species, from the aquatic fern, or from one of the flowering plants used as a control. In addition, possible structures such as trichomes were investigated. The aquatic fern species included in the study was the only species that shown these structures, but uses them for flotation purposes, rather than as defensive weapon. Water and silica content analyses complete the data collected in this chapter. Silica concentration is quite variable, intra- and interspecifically speaking. It was not possible to detect a significant difference between different plants. Moreover, no correlation between the content of this element could be established. On the other hand, it was possible to differentiate in a statistically significant way the species of ground ferns with a lower water content than the rest of the plants used. This suggests, however, that these physical defenses are not involved in the lower plants' strategy to combat herbivores.

The next chapter was dedicated to the chemical defenses. For this part, only two species of pteridophytes were used, namely a lycopod and a fern. To carry out the investigations of chemical defenses, extractions were carried out using different solvents, such as water combined with 0.1% formic acid, methanol, and dichloromethane. The extracts obtained were dried and mixed with artificial diet. Those supplemented diets were then used to feed polyphagous caterpillars. These insects had difficulty gaining weight and developing on two of the extracts made with the fern species. In a second step, fractions were made from the worst extract, the aqueous one. The fractions were again added to artificial diet and offered to the caterpillars. One of the fractions was found to be more toxic than the others, and contained phloroglucinols. This is a class of compounds known to be involved in the defense of plants like eucalyptus. It was therefore concluded that these molecules might play the same role for the fern species studied here.

In the fourth chapter of this thesis, phytohormones and volatile organic compounds (VOCs) emitted by a fern species after attacks by specialized aphids (*Idiopterus nephrelepidis*) or caterpillars (*S. exigua*), or mechanical damage were investigated. It was not possible to detect a hormonal response of the plants. Change in the VOCs emitted as a result of the aggression by the aphids were, as well, not detectable. On the other hand, it was shown that this fern species changes its odor when consumed by caterpillars or when subjected to wounds alone, both cases being discernible. This mechanism is therefore not limited to flowering plants and could be useful for pteridophytes. We can hypothesize that they would attract beneficials such as parasitoids, helping them to overcome herbivores’ attacks.

Overall, the results of this PhD demonstrate that the study of herbivory in lower plants is complex and deserves research attention, while highlighting the importance of studying these plant groups from an evolutionary perspective. Moreover, these plants are called “primitive”, but their present-day representatives encounter the same constraints as the so-called higher plants. The early land plants are as successful and adapted to their environment as the flowering plants.

Key-words: Early land plants, Herbivory, Plant-insect interactions, Bryophytes, Pteridophytes

Table of content

1 General introduction

1.1 Biological interactions

Biological interactions are extremely diverse, complex, and play a major role in global ecosystems functioning. Understanding such complexity is of a main importance in biodiversity preservation, which is one of the major challenges of the present time.

There are many interactions involving living beings and they can be characterized by the effects they have on each organism involved. Those effects can be positive, neutral or negative for the protagonists concerned. As every combination is possible (Table 1), a double positive effect leads to an interaction called mutualism. On the opposite, the antagonism, or competition, shows a negative effect on both organisms involved. Between those extremes, combinations of beneficial and nocuous effects can be observed (Aladjov, 2011, Bronstein, 1994).

Table 1: Interactions between species ("+" means that the effect is positive for the species, "-" means that this effect is negative and "0" means that there is no effect on the species) from Bronstein (1994).

		Species 2		
		+	-	0
Species 1	+	Mutualism		
	-	Predation / Parasitism	Competition	
	0	Commensalism	Amensalism	Neutralism

1.2 Herbivory

Among biological interactions, herbivory is a particular case because even though pollination is a mutualistic relationship of herbivory, most of the herbivory relationships enter the predation/parasitism category, in which an animal eats an autotrophic organism (*i.e.* plants, algae) (Campbell and Reece, 2002). The plants are evolutionary pressed to choose the better option, according to their fitness between all the ones possible to deal with the attackers (Stamp, 2003). There are two main options if a plant is eaten by an herbivore (Fig. 1): i) the plant needs to tolerate herbivory, or ii) the plant has to defend its tissues as far as possible (Rosenthal and Kotanen, 1994). If they use the second option, the defensive strategy can be based on constitutive or inducible elements (Chen, 2008). Plants can rely on mechanical defenses, with adapted physical tissue proprieties (Lucas, 2000). For instance, some particular structures (*e.g.* spines, trichomes) and complex organic polymers (*e.g.* silica, cellulose, lignin) might be used as a protection (Rosenthal and Kotanen, 1994, Hanley et al., 2007). Chemical defenses can also be constitutive (*e.g.* toxins (Bennett and Wallsgrove, 1994, Wink, 2003)).

The plants can also use indirect defenses, mediated by the emission of volatile organic compounds (VOCs). For example, when a caterpillar eats a maize leaf, the attacked plant produces a typical molecules blend attracting parasitic wasps that lays eggs in the caterpillar, fact that interrupt the life cycle of the herbivore (Turlings et al., 1995, Turlings et al., 1990). This phenomenon is also observed belowground, when roots of maize plants are eaten by a larvae of a coleopteran species, the plant release (*E*)-β-caryophyllene that attract enthomopathogenic nematodes, which parasite the larvae and kill it before it becomes an adult beetle (Rasmann et al., 2005). Moreover, some VOCs are used in priming to prevent herbivory on neighboring plants, like indole emitted by maize plants under herbivore attack and that shows a priming effect on surrounding maize plants (Erb et al., 2015).

The defense's induction can occur thanks to phytohormonal pathway (Erb et al., 2012, Checker et al., 2018). The chemical induction happens in a relative short amount of time, whereas the macrohpysical defenses like trichomes are induced later in time and in new leaves (Barton, 2016). Of course, all those different defensive strategies and mechanisms can by combined in a plant species to overcome the herbivores' attacks (Agrawal and Fishbein, 2006).

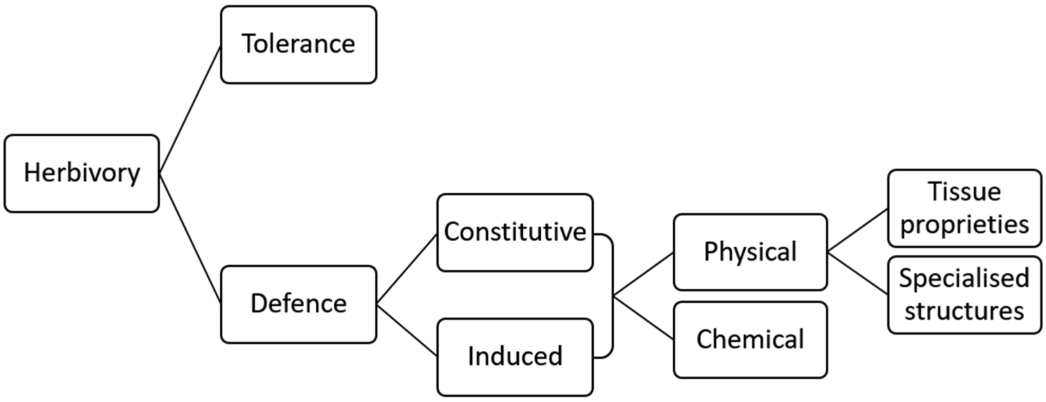

Fig. 1: Diagram of possible strategies used by plants to face herbivory.

On the other hand, lack of nutrients inside plant tissues can also be a reason why herbivores do not consume some plant species. In poor environments, the plants tend to invest more in defenses than in rich environments, leading to a correlation between slow growth rate and highly defended plants (Coley et al., 1985). Nevertheless, some specialized herbivores (*i.e.* feeding on a single or a reduced number of species) are able to eat well-defended plants. It requires adaptations such as particular mouthparts, detoxification pathways, sequestration of plant toxins, etc. For instance, many specialized insect species accumulate toxic secondary metabolites and use them for their own defense against predators (Müller et al., 2001). For example, *Diabrotica* spp. (Coleoptera: Chrysomelidae), are able to feed on Cucurbitaceae plants, which produce cucurbitacins (*i.e.* oxygenated tetracyclic triterpenes toxic to most herbivores) and sequester those toxic compounds and become toxic to their predators (Ferguson and Metcalf, 1985).

1.3 Plant evolution and primitive plants

Evolutionary studies showed that bryophytes *sensu lato* are the earliest plants that came out of the water to colonize land habitats during Ordovician (*ca.* 449 Ma) (Clarke et al., 2011).

During the colonization of lands by plants, successive steps have been distinguished. The first embryophytes, which is the monophyletic group containing all the terrestrial plants, had to face many new stressing factors (*i.e.* UVs from direct sun light, air contact, gravity) (He et al., 2013). In particular, the UVs dilemma generates a the evolution of many of the actual pigments present in plants (Rozema et al., 2002). Some other apomorphies, these novel features from a group of living organisms (clade), also appeared thanks to selective constraints like competition for sunlight exposure (Horn, 1971).

There is still a debate to know which clade in Embryophyta (*i.e.* land plants) is the more basal one, based on comparison between morphological and molecular data, but most of the studies agree on liverworts (Hepaticophyta) to be that first land plant clade. After that, the mosses, or bryophytes *sensu stricto* (Bryophyta) evolved, followed soon by the hornworts (Anthocerotophyta). Afterwards, the first vascular plants appeared with the lycophytes group (Lycophyta), then with the monilophytes (Monilophyta) containing horsetails and ferns. Finally, the most recent group of land plants emerged with the seed plants (Spermatophyta) gathering successively gymnosperms and angiosperms (Qiu et al., 2006, Ruhfel et al., 2014, Nickrent et al., 2000) (Fig. 2).

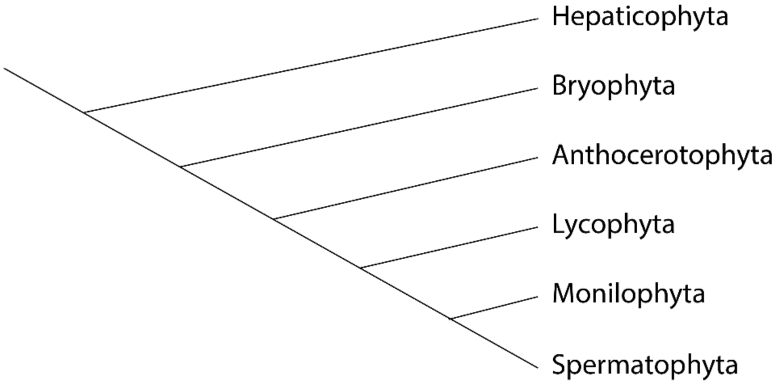

Fig. 2: Cladogram of Embryophyta taxa

1.3.1 Bryophytes *sensu lato*

The bryophytes is a paraphyletic group (*i.e.* group which does not contain all the ancestors and/or all the descendants of a taxa) that is divided in three earliest monophyletic taxa (*i.e.* group containing all the ancestors and all the descendants of a taxa): Hepaticophyta (liverworts), Bryophyta (mosses), and Anthocerotophyta (hornworts) (Shaw and Renzaglia, 2004). It is difficult to find and to identify fossils of Bryophytes, because of the softness of their tissues (Kenrick et al., 2012), but the oldest Hepaticophyta are found in Devonian strata (Givetian ca. 388 Ma) (Hernick et al., 2008). Bryophytes were also identified in the Carboniferous strata dated from 330 ± 4 Ma (Hübers and Kerp, 2012). Around 12’800 still existing species of mosses and sphagnum have been described, although this number has slightly decreased after recent taxonomic revisions (Crosby et al., 2000). Despite available morphological and molecular data, there is still debated which Embryophyta clade (i.e. land plants) is the most primitive. Yet, most recent studies suggest that liverworts (Hepaticophyta) represent the most ancestral species. The mosses (Bryophyta) evolved next, followed by hornworts (Anthocerotophyta) (Qiu et al., 2006).

1.3.2 Pteridophytes (*sensu* Pryer *et al.* (2004))

The “pteridophytes” form a paraphyletic group as well, containing the two earliest monophyletic taxa of vascular plants: lycophytes and monilophytes (*sensu* Pryer et al. (2004)). According to phylogenetic studies, the lycophytes are the first vascular plants found in the evolution tree. In this group, we can distinguish three taxa: clubmosses (Lycopodiopsida), quillworts (Isoetales order with *Isoetes* as the only extant genus left) and spikemosses (Selaginellales order with *Selaginella* as the only extant genus) (Pryer et al., 2004).The other main group that evolved after the lycopods are the monilophytes, which contain ferns (Psilotales order), ophioglossoid ferns (Ophioglossales order with Ophioglossaceae as the only extant familly), horsetails (Equisetopsida class with Equisetales as the only extant order), marattioid ferns (Marattiales order), and leptosporangiate ferns (Polypodiales order) (Pryer et al., 2004).

After the apparition of the first land plants, the bryophytes *s.l.*, and the evolution of plants with a vascular system in the pteridophytes, the more recent group of terrestrial plant appeared. This group is the seed plants (Spermatophyta) are the most modern land plants, comprising both gymnosperms and angiosperms (Ruhfel et al., 2014, Qiu et al., 2006, Nickrent et al., 2000).

1.4 Herbivory in early land plants

The primitive plants are assumed to not be consumed by herbivores for a long time (Ehrlich and Raven, 1964, Hendrix and Marquis, 1983, Prins, 1982). The more common hypothesis to explain this phenomenon is that those plants do not contain enough nutrients. Moreover, mosses would only be eaten in cold or poor environments (Crafford and Chown, 1991, Butet, 1990).

Some exceptions are found in the literature with specialized herbivores, able to successfully develop on primitive plants. For example, larvae of crane flies (Tipulidae) are known to use mosses as food and shelter (Smith et al., 2001) and sawfly species (Tenthredinidae, Hymenoptera) are laying eggs in new fern fronds so their larvae feed on (Schreiner et al., 1984).

Even if some studies are trying to provide some information about the relationship between primitive plants and herbivores (Markham et al., 2006), many questions remain and the answers could tell us a lot about evolution of plant herbivory, by knowing the mechanisms employed by early land plants.

1.4.1 Tolerance strategy

Tolerance of herbivory pressure is a strategy used in plants and consist of limiting the negative consequences of herbivory on their own fitness (Rosenthal and Kotanen, 1994, Strauss and Agrawal, 1999). This strategy has been poorly investigated in early land plants. One study investigating peatlands mosses showed that they are more likely to use resistance and defensive strategy against herbivores (Chen et al., 2021). Regarding ferns, Farias et al. (2020) concluded that the tropical species they investigated do not use tolerance but rather defense or low nutrition as main strategy against herbivore, rather than tolerance.

1.4.2 Physical defenses

The role of physical defense in plants can be played by macroscopic as well as microscopic features. The first ones are for example trichomes, which can be either non-glandular or glandular. While the first type can be found in all terrestrial groups, the second requires a vascular system and is then not found in bryophytes (Wagner et al., 2004). Touch sensitive trichomes were present in fossil seed-ferns and are then probably shared with all seed plants (Krings et al., 2002). In modern ferns, trichomes are recorded in the investigated species but do not exhibit a consistent pattern liked to defense against herbivores (Farias et al., 2020).

On the other hand, microscopic physical traits can be found in both bryophytes and pteridophytes groups. Macromolecules contained in the cell wall (*e.g.* cellulose, lignin) can be effective towards herbivores (REF). Another option is the use of silica (*i.e.* silicon dioxid) incrustations in plant tissues that would damage herbivores' mouthparts and digestive tract (Vicari and Bazely, 1993). One review on silica investigation in plants highlighted the lack of information in early land plant groups, bryophytes and pteridophytes being largely less investigated than seedplants (Hodson et al., 2005).

Additionally, water content is also liked to herbivores' performances with a correlation between low success of the consumers and low water content (Poorter et al., 2004, Schädler et al., 2003). Water content is known in bryophytes to be variable as they are highly desiccation tolerant (Proctor, 2001, Wood, 2007). The ferns from tropical environment has been demonstrated showing low water content that negatively affected herbivores (Farias et al., 2020).

1.4.3 Chemical defenses

The herbivores often have to face chemical compounds synthetized by plants to fight them. Those molecules belong to primary metabolites, like proteins, as well as secondary metabolites (Erb and Kliebenstein, 2020). In early land plants, we can find different substances that can interfere with herbivores, including proteins (Markham et al., 2006). On the secondary metabolites' side, there are in the bryophyte group oxylipins and terpenes in liverworts (Croisier et al., 2010, He et al., 2013, Asakawa et al., 1983), and phenols and fatty acids in mosses (Davidson et al., 1990, Davidson et al., 1989, Glime, 2006, Parker et al., 2007). Regarding the pteridophytes, more substances have been studied like alkaloids in lycopods (Aver and Trifonov, 1994), and many classes in the fern group like phenols, flavonoids and cyanogenic glucosides for example (Bohm, 1968, Bohm and Tryon, 1967, Star and Mabry, 1971, Schreiner et al., 1984).

1.4.4 Inducibility of the defenses

In plants, chemical defenses as well as physical ones are inducible and this induction of the defenses relies on hormonal pathways (Erb et al., 2012, Checker et al., 2018). Salicylic acid (SA) and jasmonic acid (JA) are the two main phytohormones involved in plants' defensive induction against herbivores. SA is more likely to be used in case of attack by phloem feeders, and JA by chewing ones. There is a SA-JA crosstalk that is supposed to regulate the hormonal reaction in a given individual (Thaler et al., 2012). A direct link between both SA and JA and VOCs production has been established (Ozawa et al., 2000). Indeed, VOCs can be constitutively emitted, but are also often released upon damage (Kessler and Baldwin, 2001, Dicke et al., 2009).

Phytohomones are present in bryophytes, but their role against biotic stress is not well assessed (Záveská Drábková et al., 2015). The pteridophyte group has been studied a bit further on this aspect and been tested in the context of herbivory, showing sometimes a response comparable to what is observed in higher plants, like an increase of VOCs emitted by the fern (Boland et al., 1995, Radhika et al., 2012, Živković et al., 2021, Kessler et al., 2015b, Soriano and Clavijo-McCormick, 2020).

1.5 Plant models used

In this thesis, a large spectrum of early land plants was investigated. The 17 species used are presented in Table 2, each of them coming from different families and distributed as follow: two species of liverworts, 6 mosses, one clubmoss, 7 ferns (including one horsetail) and 2 flowering plants were used as control plants. The origin of the specimen used is mostly local but to ensure as much diversity as possible, some species were purchased in garden center or online. The botanic garden of Neuchâtel also helped by providing access to some of the species.

Table 2: Species used and their respective family, phylum and functional group mentioned in the thesis

Species	**Family**	**Phylogenetic division**	**Functional group**
Marchantia polymorpha	Marchantiaceae	Hepaticophyta	Bryophytes *s. l.*
Riccardia chamedryfolia	Aneuraceae		
Fontinalis antipyretica	Fontinalaceae	Bryophyta	
Anomodon viticulosus	Anomodontaceae		
Rhytidiadelphus triquetrus	Hylocomiaceae		
Ctenidium molluscom	Hypnaceae		
Pseudoscleropodium purum	Brachytheciaceae		
Lycopodium annotinum	Lycopodiaceae	Lycophyta	Pteridophytes
Equisetum scirpoides	Equisetaceae	Moniliophyta	
Salvinia natans	Salviniaceae		
Adiantum venustum	Pteridaceae		
Asplenium trichomanes	Aspleniaceae		
Polypodium vulgare	Polypodiaceae		
Polystichum aculeatum	Dryopteridaceae		
Dryopteris filis-max	Dryopteridaceae		
Zea mays	Poaceae	Spermatophyta	Spermatophytes
Lactua sativa	Asteraceae		

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2 Aim of the thesis and hypothesis

The aim of this thesis is to fill the gap of knowledge of the herbivory on early land plants, which are bryophytes, and on early vascular ones, the pteridophytes.

The first chapter aimed to measure the nutritive value of primitive plants in terms of proteins, amino acids, sugars content. As the early land plants need the nutrients for their own metabolism and as the literature suggest that the defensive strategies are more likely to be used by mosses and ferns, we hypothesized that the levels of nutrients are not explaining the lack of herbivory in those groups of plants.

The second chapter focuses on the physical factors that could interfere with herbivory like trichomes presence, and water or silica concentration in plant tissue. We made the hypothesis that the early land plants could rely on such physical traits to ensure their defense, but more likely on microscopic ones like silica content rather than macroscopic trichomes for example.

In the third chapter, we studied the potent chemical defenses present in two pteridophytes species. Based on previous results and literature knowledge, we hypothesized that secondary compounds present in those plants could be toxic to generalist herbivores, and that specific class of chemicals could be isolated and identified.

Finally, the fourth chapter takes an interest in phytohormones and volatile organic compounds (VOCs) in a particular species of fern, subjected to different herbivores and to mechanical damage. For this aspect of plant-herbivore interactions, examples already studied showed that early vascular plants like ferns are able to emit VOCs linked to herbivores' attack and hormonal stimulus. We build our hypothesis accordingly, expecting to find a hormonal and VOCs emission induction in the fern species studied.

3 Chapter 1: Early land plants: Plentiful but neglected nutritional resources for herbivores?

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Early land plants: Plentiful but neglected nutritional resources for herbivores?

Audrey Duhin[1] | Ricardo A. R. Machado[2] | Ted C. J. Turlings[1] | Gregory Röder[1]

[1]Laboratory of Fundamental and Applied Research in Chemical Ecology, Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland

[2]Experimental Biology Research Group, Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland

Correspondence
Gregory Röder, Laboratory of Fundamental and Applied Research in Chemical Ecology, Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland.
Email: gregory.roeder@unine.ch (https://www.unine.ch/farce/home/membres/gregory-roeder.html)

Abstract

Plants and herbivores have been engaged in a co-evolutionary arms race for millions of years, during which plants evolved various defenses and other traits to cope with herbivores, whereas herbivores evolved traits to overcome the plants' resistance strategies. Herbivores may also avoid certain plants merely because these lack suitable nutrients for their development. Interestingly, the number of herbivores that attack individual early land plants like mosses and ferns is quite low. Among others, poor nutrient quality has been hypothesized to explain the apparent low herbivory pressure on such plants but still waits for scientific evidences. Here, the nutritive suitability of representative mosses and liverworts (bryophytes) and ferns (pteridophytes) for herbivores was investigated using feeding assays combined with quantifications of nutrients (proteins, amino acids, and sugars). Growth and survival of two polyphagous herbivores, a caterpillar and a snail, were monitored when fed on 15 species of bryophytes and pteridophytes, as well as on maize (*Zea mays*, angiosperm) used as an external indicative nutritional resource. Overall, our results show that the poor performance of the herbivores on the studied early land plants is not correlated with nutritional quality. The growth and performance of snails and caterpillars fed with these plants were highly variable and independent of nutrient content. These findings arguably dismiss the poor nutrient quality hypothesis as the cause of herbivory deficit in bryophytes and pteridophytes. They suggest the possible presence of early resistance traits that have persisted all through the long evolutionary history of plant–herbivore interactions.

KEYWORDS
early land plants, ferns, generalist herbivores, insect, mosses, nutritive content, snail

TAXONOMY CLASSIFICATION
Chemical ecology, Community ecology

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1 | INTRODUCTION

Evolutionary studies have shown that bryophytes are the earliest plants that moved from their aquatic habitats to colonize terrestrial ecosystems during Ordovician (ca. 449 million years ago (Ma); Clarke et al., 2011). The first land plants had to face and adapt to many new stress factors such as ultraviolet radiation (UV) from direct sunlight, risk of desiccation, and gravity (He et al., 2013; Horn, 1971; Rozema et al., 2002). Bryophytes are divided into three groups: Hepaticophyta (liverworts), Bryophyta (mosses), and Anthocerotophyta (hornworts; Shaw & Renzaglia, 2004). Later, the first vascular plants evolved in the paraphyletic pteridophytes group (sensu Pryer et al. (2004)) with the clubmosses (Lycophyta), and both the horsetails and the ferns (Monilophyta). Nowadays, seed plants (Spermatophyta) gather all the gymnosperms and angiosperms (Nickrent et al., 2000; Qiu et al., 2006; Ruhfel et al., 2014).

Herbivory is a ubiquitous phenomenon that affects most seed plants (Marquis, 1992). By contrast, bryophytes and pteridophytes are generally considered to be largely spared from such antagonistic interactions (Cooper-Driver, 1978; Gerson, 1969; Glime, 2006; Hendrix, 1977; Soo Hoo & Fraenkel, 1964). Although they lack tough tissues like wood or cork, these apparently fragile and readily accessible plants are mostly avoided by modern herbivores. This may explain why few studies have been carried out on the consumption of mosses and ferns by herbivores (Haines & Renwick, 2009). For decades, it has been hypothesized that early land plants are unsuitable hosts for herbivorous insects, lacking crucial nutrients (namely the poor nutrient quality hypothesis, Ehrlich & Raven, 1964; Hernick et al., 2008; Prins, 1982). Deterring secondary metabolites and digestive chemical or physical inhibitors would be the alternative hypotheses explaining this apparent dislike (Haines & Renwick, 2009). In 1964, Ehrlich and Raven stated that among herbivorous lepidopteran larvae, none is known to feed on bryophytes, nor on ferns, before mentioning the fern eaters *Papaipema* moths (Noctuidae; see page 598 in Ehrlich & Raven, 1964). Years later, studies on early land plants reported that various herbivores, including mammals, birds, gastropods, and arthropods can actually feed on bryophytes (Davidson et al., 1990; Fang & Zhu, 2013; Glime, 2017; Maciel-Silva & Santos, 2011; Singer & Mallet, 1986) and pteridophytes (Hamm & Fordyce, 2016; Schneider, 2016). Some insects have even been shown to specialize on mosses, like weevils from the tribe Ectemnorhinini (Coleoptera: Curculionidae; Chown, 1990), larvae of some snipe flies (Diptera: Rhagionidae; Imada & Kato, 2016), or crane flies species (Diptera: Tipulidae; Freeman, 1967). In this context, Smith et al. (2001) evaluated the development of crane flies on five different moss species, finding that all larvae gained weight and seemed to choose which plants were of the best quality. Yet they concluded that herbivores probably select bryophytes as shelter rather than as food, because crane fly larvae still preferred angiosperm leaves, if available. Noticeable exceptions to fill the gap in our knowledge on herbivory in early plants were works of Markham et al. (2006), which assessed protein-based defenses against phytophagous insects in several ferns and mosses, of Haines and Renwick (2009), which suggested that some preingestive mechanisms might be crucial in deterring herbivory on mosses rather than nutrient poverty, and both of Hendrix and Marquis (1983) and Patra and Bera (2007) showing that damages caused by herbivory in three, and 11, respectively, tropical fern species can be compared to that found for angiosperms. In cold and heathland environments, only generalist herbivores have been observed to feed on mosses (Butet, 1990; Crafford & Chown, 1991).

It is relevant to point out that mosses lack hard supporting sclerenchyma and resulting stiff vascular tissues (i.e., phloem and xylem). Despite some fossil-based evidence for arthropod herbivory on liverworts from the Middle Devonian (Labandeira et al., 2014), it has been suggested that these soft tissues are less prompt to fossilize, compared to that of vascular plants (Kenrick et al., 2012), making difficult to properly document the 400 Ma old interactions between mosses and herbivores. Records of leaf damage on fern fossils seem to be more common. For clubmosses and ferns, Paleozoic indications of herbivory can be seen from the Late Silurian to Early Devonian (417 to 403 Ma ago; Labandeira, 2007) or from Triassic (252 to 201 Ma ago; Imada et al., 2022) and the Late Miocene (10.29 to 5.27 Ma ago), which has firmly been attributed to insects (Robledo et al., 2015). Nowadays, few insects are specialized in ferns, but some sawfly species (Hymenoptera: Tenthredinidae) are known to lay their eggs in new fern fronds, where their larvae can feed on fresh tissues right after hatching (Schreiner et al., 1984). Mostly, ferns, like mosses, are considered to be unsuitable host plants, although few studies only really focused on their nutritional value (Buckingham et al., 1978).

Regarding the chemical defenses in early land plants, liverworts have been shown to contain defense compounds like oxylipins (Croisier et al., 2010; Ponce de Leon et al., 2015; Rempt & Pohnert, 2010), terpenes, aromatics (He et al., 2013), and sesquiterpene lactones (Asakawa & Takemoto, 1979; Knoche et al., 1969). In mosses, phenols have been found to accumulate in gametophytic cell walls (Davidson et al., 1989, 1990) and show repellent effects on gastropods and isopods (Glime, 2006). The aquatic moss *Fontinalis novae-angliae* contains fatty acids that repel insects (Parker et al., 2007). In pteridophytes, some clubmosses rely on alkaloids for chemical defense (Aver & Trifonov, 1994; Kitajima & Takayama, 2011). Ferns also contain phenols (Bohm, 1968; Bohm & Tryon, 1967), as well as flavonoids (Star & Mabry, 1971). The bracken fern *Pteridium aquilinum* contains cyanogenic glucosides with proven negative effects on insect development (Schreiner et al., 1984). All these defenses suggest that pteridophytes might be chemically protected; however, the exact influence on herbivores was rarely studied.

If present, trichomes seem to be the main physical defensive structures in bryophytes and pteridophytes, but their exact functions against herbivores still need to be studied. As well, some fern species are known to use extrafloral nectaries recruiting ants for protection against herbivores (Heads & Lawton, 1984; Koptur et al., 1998).

Surprisingly, the longstanding cohabitation of herbivores with early plants does not seem to have allowed a frequent specialization on this plentiful resource. The intriguing relationships between these plants and herbivores remain poorly understood. Further insight into these relationships could contribute to a better understanding of the early evolution of plant defenses and how they may have been maintained and adapted to changing biotic and abiotic environments (Markham et al., 2006).

Combining feeding experiments and nutritional quality assessments, our study aims to test the hypothesis that bryophytes and pteridophytes are of poor nutritional quality, which could explain, for a part, the possible low herbivory pressure on these plants. Both the survival and developmental performance of two polyphagous herbivores were monitored in feeding experiments using representative species of mosses, liverworts, and ferns, as well as one angiosperm species (maize, *Zea mays*), used as an external indicative nutritional resource. The prime objective of assessing the nutritive suitability of a representative panel of early land plants for generalist herbivores excludes an exhaustive comparison of the nutritional values shown by all the major plant taxonomic groups. In this context, sugar, protein, and amino acid contents of leaves were quantified with chemical analyses. By linking herbivore performance and plant nutritional quality, this study offers a general overview of the early land plants' suitability for the two herbivores. Our results bring to the fore that the observed herbivores' performances are not driven by nutritional quality, predicting a more important role for chemical or physical defensive mechanisms than commonly assumed in both bryophytes and pteridophytes.

2 | MATERIAL AND METHODS

2.1 | Plants

Wild plants were collected in and near the canton of Neuchâtel (Switzerland). If not available in their natural habitat, they were grown in the botanical garden (Jardin Botanique, Neuchâtel) or purchased in a specialized garden center (Flower Market Dietrich, GmbH). Two weeks before each experiment, all plants were acclimatized at the University of Neuchâtel in a plant growth chamber (CLF Plant Climatics) with the following conditions: 15°C; 70% RH; day/night photoperiod of 12:12 h (light:dark). All plant species used for feeding experiments and chemical analyses belong to the following three groups: (1) bryophytes with two liverworts species (*Marchantia polymorpha* and *Riccardia chamedryfolia*) and five mosses species (*Fontinalis antipyretica*, *Rhytidiadelphus triquetrus*, *Pseudocleropodium purum*, *Ctenidium molluscum*, and *Anomodon viticulosus*); (2) pteridophytes including one clubmoss species (*Lycopodium annotinum*), seven monilophytes (one horsetail: *Equisetum scirpoides*; and 6 ferns: *Salvinia natans*, *Adiantum venustum*, *Asplenium trichomanes*, *Polypodium vulgare*, *Polystichum aculeatum*, and *Dryopteris filix-mas*); and (3) one external indicative angiosperm species with maize (*Zea mays* var. Delprim, 3 weeks old after germination, 3 leaves stage, grown in the plant growth chamber under the same conditions as other plants).

2.2 | Herbivores

The two polyphagous herbivores used were second instar caterpillars of the beet armyworm *Spodoptera exigua* (Insecta, Lepidoptera: Noctuidae) reared at the University of Neuchâtel, and wild adults of *Cochlicella barbara* snail (Gastropoda, Pulmonata: Helicidae) collected near Montpellier (France). They all were kept under controlled conditions (23 ± 1°C; day/night photoperiod of 12:12 h) in growth chambers and were fed either with a lepidopteran artificial diet for caterpillars or with fresh lettuce for snails aiming to avoid any habituation for the plants tested.

2.3 | Feeding and performance bioassays

During each trial, one individual caterpillar or snail was placed in a plastic box (5 cm diameter; 2 cm height) and provided with one of the tested fresh food ad libitum (no-choice food test). For each plant species, 4 distinct colonies (mosses) or individual shoots (ferns) were used as food providers. A total of 40 herbivores (20 caterpillars and 20 snails) were allowed to feed on one of the 16 diet types during the bioassays. Each herbivore was weighed every 3 days and the developmental stage of the *S. exigua* caterpillars was recorded (i.e., larva, pupa, or imago). The experiment was carried out until caterpillars died or when adults emerged. The *C. barbara* snails were allowed to feed for 33 consecutive days before ending the assay. To maintain proper humidity levels a moistened piece of filter paper, for the caterpillars, or a piece of wet plastic sponge, for the snails, was placed in the plastic boxes. The bioassays were conducted under controlled laboratory conditions (artificial light with a 12:12 h (light:dark) cycle, constant 23 ± 1°C temperature). Aiming to verify that the experimental setup cannot by itself negatively influence the performances of herbivores, a commercial artificial diet designed for caterpillar rearing was added as a control, leading to a maximum of 17 different diets during the feeding and performance bioassays. The nutritive content of this artificial diet was not assessed for further comparison.

2.4 | Nutrients quantification

Each class of nutrients (i.e., proteins, amino acids, and sugars) was measured on four different individual colonies (mosses) or shoots (ferns) per plant species ($n = 4$). For each entity, the average values from three technical replicates were combined as one biological replicate, which was used in all further statistics.

2.5 | Proteins

Soluble proteins were extracted from 20 mg of fresh leaves using 400 µl of an SDS buffer (sodium dodecyl sulfate 2%), Tris HCl (hydrochloride) (pH 8) 100 mM, NaF (sodium fluoride) 10 mM and PIC reagents (Paired-Ion Chromatography reagents 2.5 mM diluted in water). Samples were incubated for 30 min at 37°C in a dry bath. Then, extracts were centrifuged for 15 min at maximum speed at room temperature (22°C). To quantify proteins, 1 to 6 µl of the resulting supernatants (depending on the concentration) were added to 1 ml of the reactant mixture from the BCA (bicinchoninic acid) Protein Assay Kit (Pierce, Thermo Scientific) and incubated again for 30 min at 37°C. For quantification, the absorbance of the solution was measured at 562 nm, then compared with a calibration curve prepared with pure BSA (bovine serum albumin). Minor adjustments in terms of extract quantities were made in order to use the classic protocol developed for angiosperms by Stout and Al-Niemi (2002). The reproducibility of the method was validated by tri-replicates on the same sample with a coefficient of variation (or relative standard deviation) kept under 15%. As described above, three technical replicates per sample were analyzed.

2.6 | Total amino acids

Chemical analyses of total amino acids (i.e., free and those fixed in proteins) were carried out at the Service "Biomass and Green Technologies" (University of Liège—Gembloux Agro-Bio Tech), following the method described in Vanderplanck et al. (2013). One milliliter of hydrolysis solution (6 N HCl, 0.1% phenol, and 500 µM norleucine) was added to 3–5 mg (dry weight) of plant material. The tube was placed under nitrogen for 1 min to avoid methionine degradation and then incubated for 24 h at 110°C. The hydrolysate was evaporated until dryness under vacuum in a boiling bath at 100°C. Afterward, 1 ml of sodium citrate buffer at pH 2.2 was added. The sample solution was mixed and poured into an HPLC vial after filtration (0.2 µm). Each amino acid was measured separately with an ion exchange chromatography (Biochrom 20 Plus Amino Acid analyzer). A postcolumn ninhydrin reaction produced colored derivatives, which were monitored via a UV detector, with norleucine used as the internal standard. Using this method, 16 amino acids were quantified: Asp, Thr, Ser, Glu, Pro, Gly, Ala, Cys, Val, Ile, Leu, Tyr, Phe, His, Lys, and Arg. As for the proteins, three technical replicates per sample were analyzed.

2.7 | Free and stored sugars

Extraction and quantification of both free (glucose, fructose, sucrose) and stored sugars (starch) were carried out following the method of Machado et al. (2013), with minor adaptations in the incubation steps. Soluble sugars were extracted from plant tissues using 80% (v/v) ethanol, followed by an incubation step (15 min at 80°C), with regular shaking. Pellets were re-extracted twice with 50% (v/v) ethanol (15 min at 80°C). Supernatants from all extraction steps were pooled together. Afterward, sucrose, glucose, and fructose were quantified enzymatically as described by Velterop and Vos (2001). The remaining pellets were used for an enzymatic determination of starch (Smith & Zeeman, 2006). As for the other studied nutrients, three technical replicates per sample were analyzed.

2.8 | Statistical analysis

Statistical analyses were carried out in R studio (R version 3.4.3). For leaves protein and total amino acid contents, one-way ANOVAs were performed after $\log(x \pm \alpha)$ transformation of the raw data. The logtrans function (package MASS) was used to determine optimized (α) for each variable. Tukey post hoc tests (honestly significant difference) were used hereafter to know which pairs were significantly different, and lettering was added to statistically sort the plant species onto the figures. When ANOVA assumptions failed, like for the relative amounts of amino acid and total sugar contents, nonparametric Kruskal–Wallis tests were carried out. Both perMANOVA (permutational multiple analysis of variance) and pairwise analyses were used for detailed comparisons between amino acid contents in the plant species (Anderson, 2001). Aiming to highlight likely differences between all the studied plants, principal component analyses (PCA) were carried out on amino acids and sugars. Finally, linear regressions were realized between every nutrient parameter and the developmental performances of the two generalist herbivores, with coefficients of determination (R^2) calculated with the least square method.

3 | RESULTS

3.1 | Feeding and performance bioassays

Compared with *S. exigua* caterpillars fed on both artificial diet and maize plant, caterpillars fed on early land plants did not survive or develop properly (Figure 1a,b). This was highly consistent, with only one species of fern (*A. venustum*) still hosting living caterpillars after 9 days, whereas none survived beyond day 24 (Figure 1a). All caterpillars fed on primitive plants died before pupating, while 25% fed on maize (*Z. mays*) and 80% on artificial diet achieved metamorphosis into moths. Except for the only individual able to survive on *A. venustum*, all the caterpillars that gained weight were fed on maize or artificial diet, with a typical weight loss right before pupation (Figure 1b).

The polyphagous *C. barbara* snails lost weight and suffered some mortality when fed on the different plants. No exact food intake was assessed, while snails visually fed scarcely on the plants. The mass lost across time was the lowest for snails fed with horsetail (*E. scirpoides*) and maize leaves (Figure 2), whereas the most severe mass decreases were observed on liverworts species (Figure 2, *M. polymorpha* and *R. chamedryfolia*). Overall, we observed high variability in snail performances within and between the different taxonomical plant groups used as diet. While snails were able to gain mass only when fed on artificial diet, this resource, however, triggers the worst survival rate (30%), compared with all the other plant diets (survival rate from 55% to 90%).

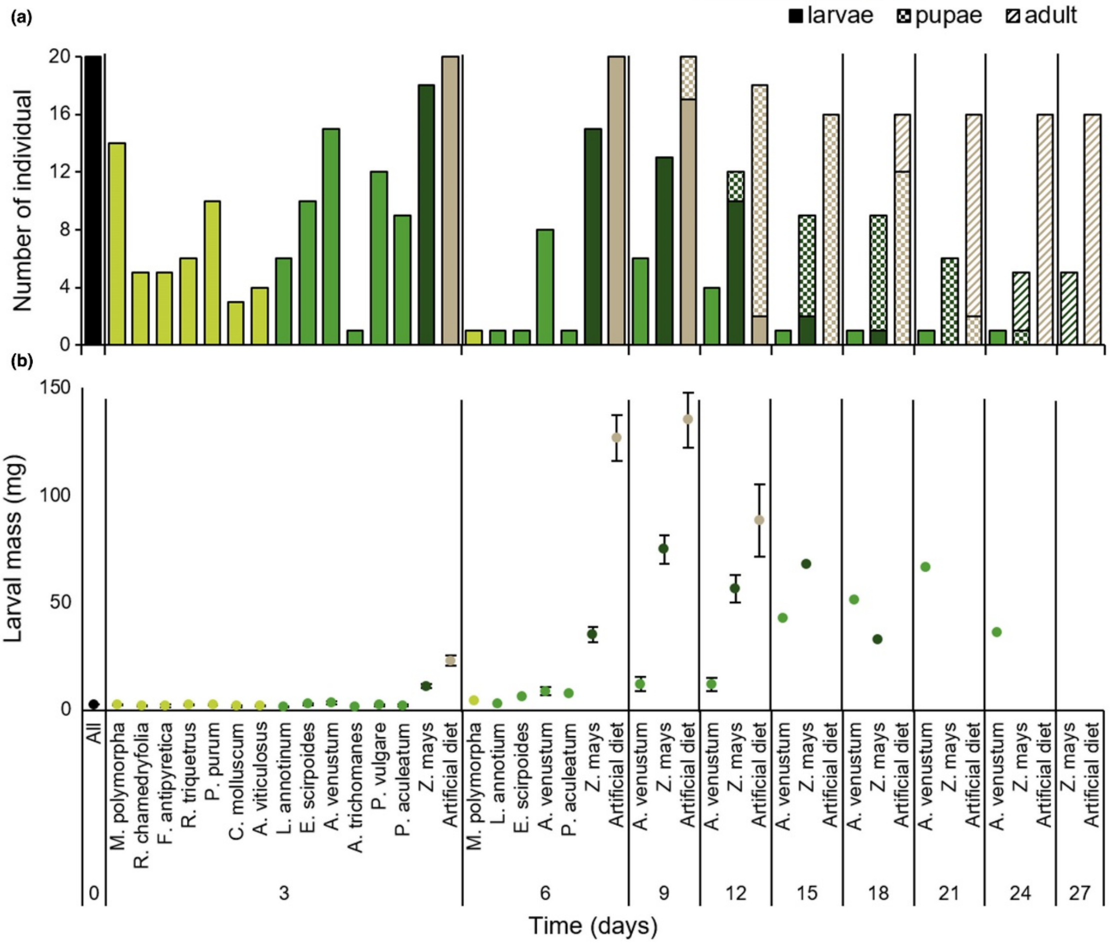

FIGURE 1 (a) Survival and developmental stage reached, and (b) larval mass monitoring of *Spodoptera exigua* caterpillars fed on primitive plants, maize, or artificial diet. Tissue of one of the 7 bryophytic (light green), 8 pteridophytic (middle green), maize (*Zea mays*, dark green) plant, or artificial diet (brown) was used to feed 20 caterpillars during the entire assays. Monitoring of individual larval mass and developmental stage was carried out every 3 days. The last adult moths had emerged from pupae on day 27, then the assays were ended. When all caterpillars assigned to one treatment died, the corresponding plant species in the figure is not shown at the next time point anymore.

Taken together, the feeding assays confirmed that generalist caterpillars and snails perform poorly when fed on the 15 bryophytes or pteridophytes included in the study.

3.2 | Nutrients quantification

3.2.1 | Proteins

Plants showed significant differences in protein contents ($F_{(15;48)} = 170.2$, p-value $< .001$; Figure 3). In the liverwort, *M. polymorpha* proteins showed similar quantities as those found in maize plants, whereas in *R. chamedryfolia* they were lower. Moss species contain lower amounts of proteins compared with maize, except for the aquatic *F. antipyretica*. In pteridophytes, the horsetail *E. scirpoides* had the lowest protein level. The clubmoss *L. annotinum* and the aquatic fern *S. natans* showed similar amounts of proteins compared with maize. All five of the terrestrial ferns (*D. filix-mas*, *A. trichomanes*, *P. vulgare*, *P. aculeatum*, and *A. venustum*) contained from three- to 10-fold higher amounts of proteins than maize plants (Figure 3).

3.2.2 | Total amino acids

In bryophytes, the liverworts *M. polymorpha* and *R. chamedryfolia*, as well as the aquatic moss *F. antipyretica*, had statistically similar

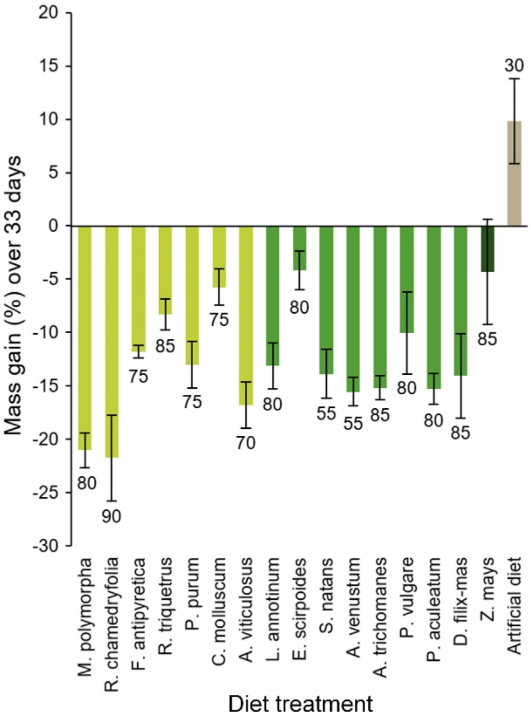

FIGURE 2 Mass gain/loss (in %) for *Cochlicella barbara* snails, fed on primitive plants, maize, or artificial diet. Tissue of one of the 7 bryophytic (light green), 8 pteridophytic (middle green), maize (*Zea mays*, dark green) plant, or artificial diet (brown) were provided during 33 consecutive days. The number next to each bar is the percent of survival after 33 days.

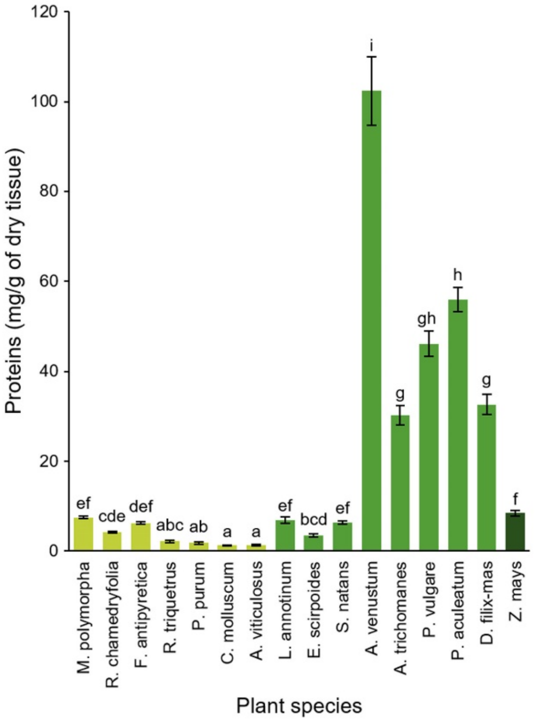

FIGURE 3 Leaf protein contents (mean ± SE, $n = 4$ for each plant species) quantified in primitive plants and maize. Tissue of one of the 7 bryophytic (light green), 8 pteridophytic (middle green), and maize (*Zea mays*, dark green) plants were analyzed. Letters above bars indicate statistical differences according to post-ANOVA Tukey test results performed with log-transformed data (ANOVA results: $F_{(15;48)} = 170.2$, p-value < .001).

total amino acid amounts as maize. In terrestrial mosses *R. triquetrus*, *P. purum*, *C. molluscum*, and *A. viticulosus*, significant lower amounts were measured (Figure 4). Total amino acid contents in mosses range typically from 100 to 170 mg/g of dry material (Figure 4), whereas protein amounts represent 5 to 10 mg/g of the dry material in this group (Figure 3). Three pteridophytic species offered a similar total amount with maize (around 160 m/g of dry material; *A. trichomanes*, *P. aculeatum*, and *D. filix-mas*), two significantly less (around 110 mg/g of dry material, *A. venustum* and *P. vulgare*), and 2 offered the fewest quantities among all the plants tested (around 60 mg/g dry material; *E. scirpoides* and *S. natans*). Ferns, in contrast to their similar or higher amount in protein than the maize plants, showed total amino acid contents that were lower or similar to those of this seed plant (Figures 3 and 4). Based on the total amounts of amino acids only, ferns showed more diverse profiles pattern than mosses (Figure 4). When relative abundances of various amino acids were considered, profiles differ slightly between species, or significantly as for the clubmoss *L. annotinum* showing the largest proportion of arginine (Arg; Figure 4).

In a principal component analysis (PCA), including all the 16 different amino acids, species tended to spread out in a way that reflects the total amino acid contents. PCA was driven by species with high levels of amino acids on one side, whereas low amino acid species clustered together in the opposite direction (cf. Figure S1). High amounts of arginine (Arg) were detected in the clubmoss species *L. annotinum* explaining the intermediate position of this species within the pool (cf. Figure S2).

As well, relative amounts of amino acids were compared in both a perMANOVA and pairwise comparisons (cf. Table S1). This statistical approach improved the comparisons between amino acid profiles. PCA carried out on these relative amounts enabled the discrimination between most of the species, even though maize and five pteridophyte species (*E. scirpoides*, *S. natans*, *A. venustum*, *A. trichomanes*, and *P. vulgare*) showed little differences in amino acid proportions (cf. Figure S2).

The relative amounts of amino acids were also used in an overall PCA comparison between the main taxonomic groups (Figure 5). The nonvascular liverworts and mosses can be distinguished from the vascular pteridophytes (i.e., clubmoss, horsetail, and ferns) and maize plant, confirming differences in amino acid patterns.

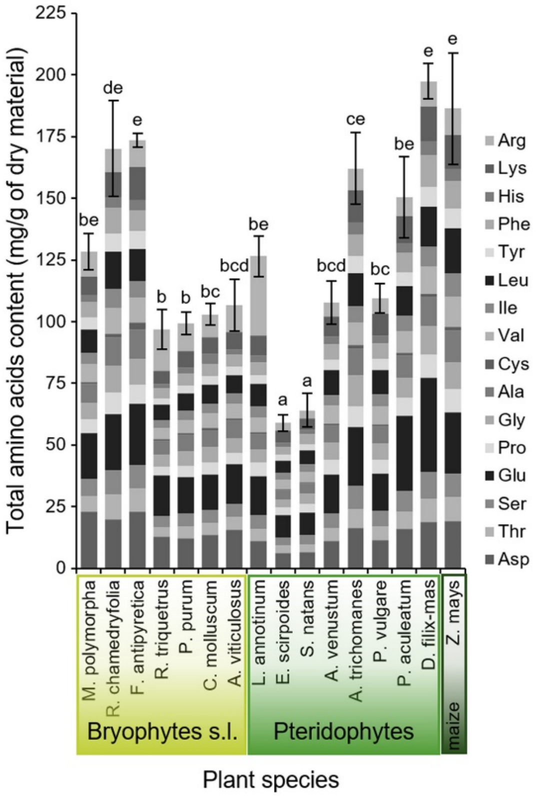

FIGURE 4 Total amino acid contents (total mean ± SE, $n = 4$ for each plant) quantified in primitive plants and maize. Tissue of one of the 7 bryophytic (light green), 8 pteridophytic (middle green), and maize (*Zea mays*, dark green) plants were analyzed. The statistics are based on the sum of every single amino acid content measured. Letters above the bars indicate statistical differences according to post-ANOVA Tukey test results performed on log-transformed data (ANOVA results: F: $F_{(15;48)} = 17.22$, $p\text{-value} < .001$). For each species, the respective amounts of 16 amino acids are shown (see labels on the right side, as follows: Asp, Thr, Ser, Glu, Pro, Gly, Ala, Cys, Val, Ile, Leu, Tyr, Phe, His, Lys, and Arg).

3.2.3 | Free and storage sugars

Sugars quantified in 16 species of bryophytes, pteridophytes, and maize varied strongly within a taxon (i.e., Hepaticophyta, Bryophyta, Lycophyta, Monilophyta). Four of the moss species had comparable levels of total sugars, whereas all pteridophytic species showed lower levels, as compared with maize (Figure 6). In order to assess properly the different quantities among the various sugar types (monosaccharides, disaccharides, polysaccharides), all quantities measured were transformed to fit with a single sugar unit taking the sugar monomers into account. The principal component analysis (PCA) carried out with these data reveals that fern profiles fall in between those measured for bryophytes and maize (Figure 7).

3.2.4 | Sugars amino acid ratios

In addition, we used the ratio between sugars and amino acid contents to identify whether some plant species provide unbalanced or unexpected profiles between these nutrients (Figure 8). Although sugar/amino acid ratios varied within both species and taxonomic groups, none of these profiles exhibited unusual patterns (Figure 8).

3.2.5 | Correlation between nutrient contents and developmental performances

Linear regressions of amino acids, proteins, sugar levels, and sugar/amino acid ratios in mosses and ferns against developmental performances of caterpillars and snails (i.e., survival time and weight gain, respectively) showed poor relationships (R^2 coefficient < 0.17, in all cases; Figure 9).

4 | DISCUSSION

Overall, we found that the developmental performance and survival of the caterpillars and snails fed on early land plants were low. Both herbivores suffered negative consequences (i.e., mass loss, extended development time, and lack of development completion) when eating proposed bryophytes and pteridophytes. Under exact same experimental conditions, they performed, however, far better on the artificial diet, and to some extent on maize leaves, that we offered as control and alternative treatments, proving that diets alone can be responsible for the poor performances observed.

Usually able to complete all larval stages and metamorphosis in 18 days under favorable conditions, beet armyworm caterpillars (*S. exigua*) were critically disadvantaged in terms of development when fed on moss and fern leaves. Beyond the third week of the experiment, only one caterpillar fed on an early land plant was still alive (i.e., on the fern *A. venustum*), whereas 80% successfully finalized their development on artificial diet and 25% on maize. Maize plants appeared to not be an advantageous host for the polyphagous *S. exigua* caterpillars. Maize is a model organism showing well-studied defensive mechanisms against herbivores (Qi et al., 2018). Here, maize was considered as an intermediate resource that herbivores may use in the field, defended but still suitable as a food supply for both caterpillars (Blanco et al., 2014) and snails (Barker, 2002). In this study, this plant cannot be seen as the inclusive spermatophyta representative in terms of nutrient contents and host suitability. As well, it is relevant to point out that before the feeding assays, all the caterpillars were fed with the nutritive artificial diet, probably contributing a part to the successful developmental rate gap observed between maize (25%) and this artificial diet (80%). Our findings confirm that moss and fern tissues are not an appropriate resource for a generalist insect, and why so few insects may exploit such plants in natural habitats. In 1980, Hendrix stated that the main fern-feeding

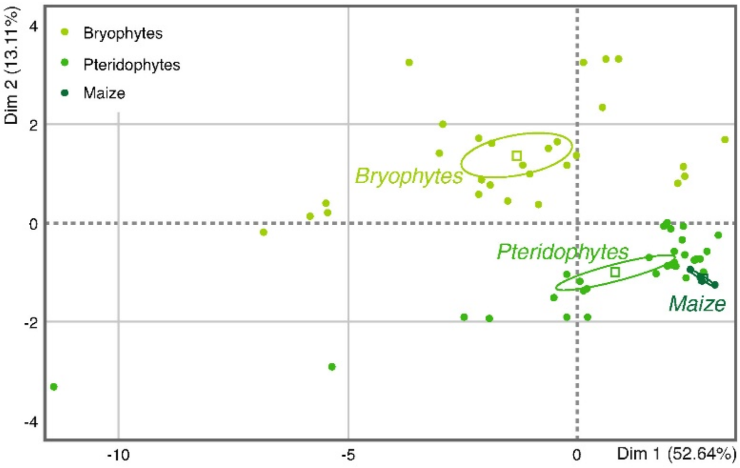

FIGURE 5 Principal component analysis clustering of the three upper plant groups studied, based on the relative amounts of 16 amino acids. The three taxa (with 95% confidence ellipses) include 16 species: 7 bryophytes (light green), 8 pteridophytes (middle green), and the additional maize plant (*Zea mays* in dark green).

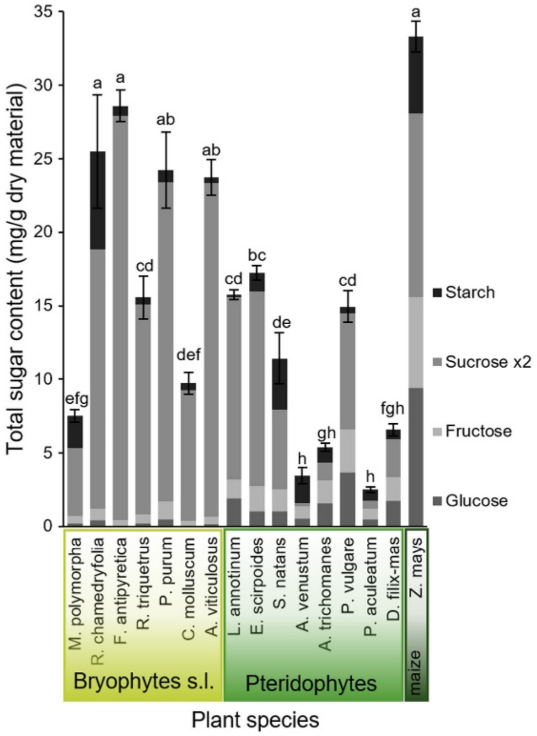

FIGURE 6 Total sugar contents (total mean ± SE, $n = 4$ for each plant) quantified in primitive plants and maize. Tissue of one of the 7 bryophytic (light green), 8 pteridophytic (middle green), and maize (*Zea mays*, dark green) plants were analyzed. The statistics are based on the sum of every single amino acid content measured. Letters above the bars correspond to post-Kruskal–Wallis pair-comparison tests (Bonferroni correction, $\chi^2 = 60.0$, p-value < .001).

insects are specialized coleopteran, hemipteran, and lepidopteran species. In natural ecosystems, he observed that the number of insect feeders on ferns was far less than theoretically expected (Hendrix, 1980). We initially hypothesized that, in a no-choice situation, the extremely polyphagous beet armyworm caterpillars with their powerful mouthparts should be readily able to feed on mosses or ferns (Azidah & Sofian-Azirun, 2006; Saeed et al., 2019). Our observations support this hypothesis and caterpillars chew and ingest plant tissues, which was visually recorded but not quantified. These results predict that beyond likely preingestion deterrence, other mechanisms impairing digestion or assimilation may occur (Haines & Renwick, 2009).

For *C. barbara* snails, the mortality rate was low when fed on early land plants, but they only gained body mass when feeding on artificial diet (9% on average for 33 days). Maize is considered acceptable as food for snails and other terrestrial mollusk species (Barker, 2002), but the gastropod tested here lost on average 4% in weight during the feeding period on this plant. Although some of the plants had stronger negative effects than maize, most of the snails were not majorly disadvantaged when feeding on the leaves of bryophytes and pteridophytes. Above all, our results express the ability of snails to starve for many consecutive days, which allows them to avoid undesirable food. Under natural conditions, such behaviors have been observed during cold, hot, and dry periods (Boycott, 1934; Cáceres, 1997; Rees, 1964). Waiting for better conditions, snails are known to go into dormancy enclosed in their shell (Pomeroy, 1968). The snail avoidance towards these diets reinforces the notion that early land plants might not be suitable host plants. Previous long-term experiments carried out on epiphytic cryptogam communities (lichens, algae, fungi, mosses) showed that gastropod grazing does not affect bryophytic diversity (Boch et al., 2016). In fact, spore dispersal by slugs may even promote bryophyte species diversity, suggesting positive outcomes of interactions between these plants and gastropods (Boch et al., 2015).

The qualitative and quantitative measurements of the nutrients provided by plant leaves failed to explain the poor performances by the herbivores. Compared with maize, concentrations of proteins in bryophytic species were either equivalent (2 species), or significantly lower (5 species). For pteridophytic leaves, they showed similar (2

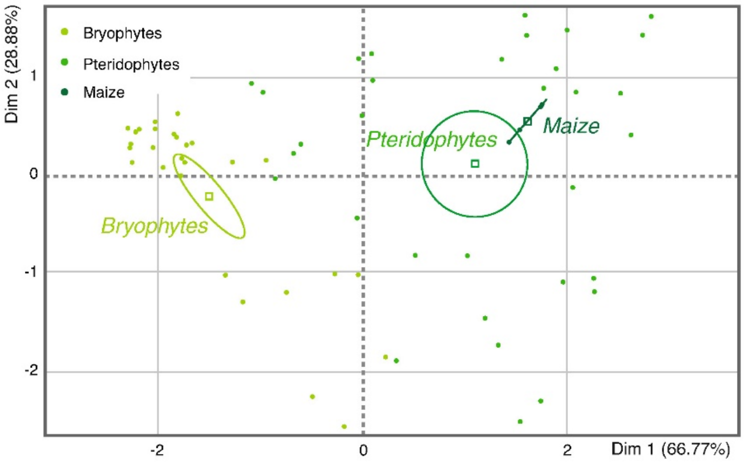

FIGURE 7 Principal component analysis based on relative amounts of sugars detected in mosses, ferns, and maize. The three upper groups (with 95% confidence ellipses) include 16 species: 7 bryophytes (light green), 8 pteridophytes (middle green), and the additional maize plant (*Zea mays* in dark green).

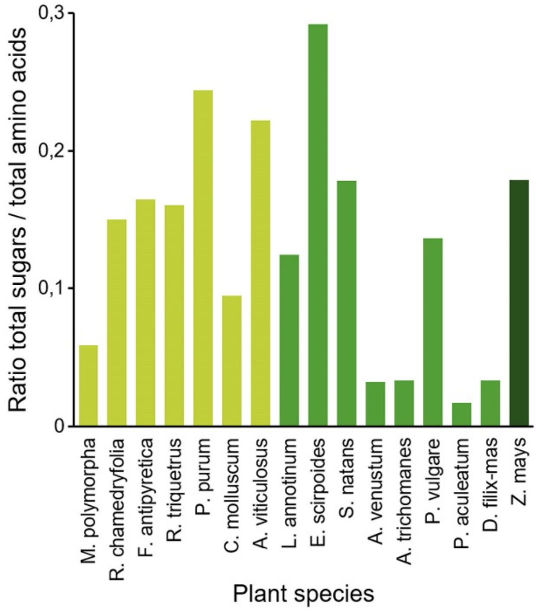

FIGURE 8 Sugar/amino acid ratios measured in primitive plants and maize. Tissue of one of the 7 bryophytic (light green), 8 pteridophytic (middle green), and maize (*Zea mays*, dark green) plants were analyzed.

species) or significantly higher concentrations (5 species), and only one of the species has a significantly lower amount. When correlating protein profiles with the developmental performance of *S. exigua* caterpillar or *C. barbara* snail, no causal connection emerged (Figure 9).

Amino acid contents showed variation between the studied plants, both in quantity and quality. Between the plant species, the amino acid patterns appeared to be mainly comparable, with few exceptions as for the clubmoss *L. annotinum* containing a higher amount of arginine. The importance of arginine in this species has not yet been studied and may warrant further investigations.

Paradoxically, if protein contents in mosses seem to be inferior to those found in pteridophytes and maize, their total amino acid contents showed to be equivalent or higher than those of many ferns. This could be explained by a high amount of free amino acids in mosses whereas pteridophytes and maize plants store these ones mainly inside their proteins. Free or fixed in proteins, none of our results suggests that amino acids may be responsible for the hypothetical poor nutritional quality of early land plants. Once again, when correlated with herbivore performance, no consistent patterns were found (Figure 9). The plant amino acid profiles, or ratios, can be of importance for insect growth (Bernays & Chapman, 1994, 2000) and interactions with plants, as shown for insect pollinators: butterflies favor nectar with high amino acid content, which drives the selection for nectar composition (Mevi-Schütz & Erhardt, 2005). Similarly, amino acids in pollen have been shown to affect flower selection by solitary bees (Weiner et al., 2010). Based on our plants' nutritional values, generalist herbivores should cope with the varying amino acid and protein levels offered by ferns and mosses included in this study. Some specialized sap-sucking aphids can develop on ferns, even though the phloem normally does not offer all of the required amino acids (Baumann, 2005). The natural fluctuations in amino acid availability and ratios can complicate feeding on mosses and ferns, but this is also the case with all other wild plants exploited by herbivores (Özcan, 2016; Watson & Creaser, 1975). To minimize possible constraints of varying nutrient contents, our plants were kept for two weeks in standard soil and under controlled conditions before feeding assays and nutrient quantifications. Interestingly, the principal component analyses based on amino acid profiles readily discriminated between the main plant taxa that were studied. Profiles for bryophytic species were distinct from those analyzed for both pteridophytes species and maize (Figure 5).

Both proteins and amino acids are of major importance for embryogenesis and the development of insect herbivores (Chen, 1966), then a lack of these primary metabolites in host plants could explain

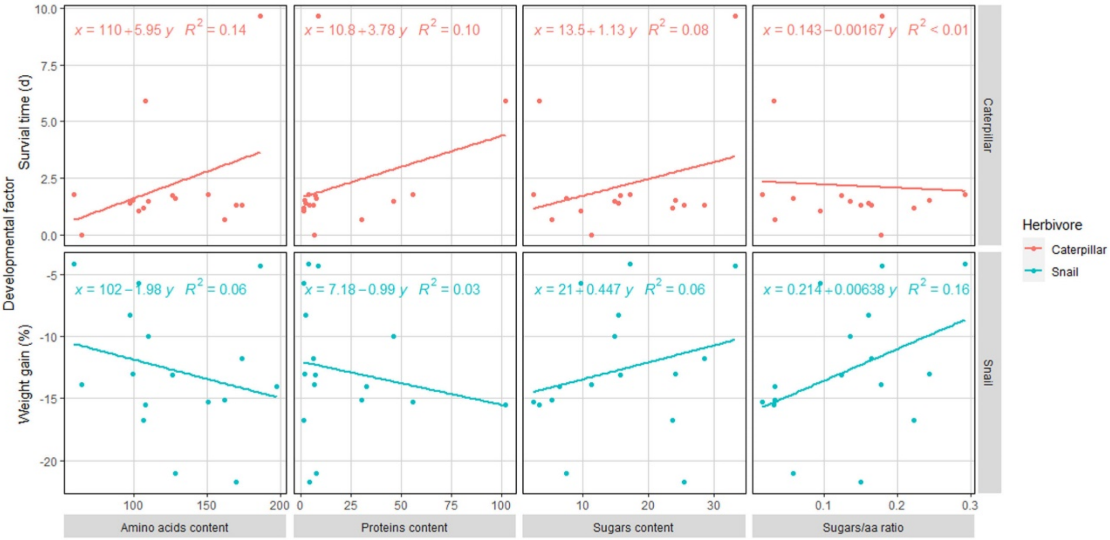

FIGURE 9 Linear regressions between nutrient parameters (amino acids, proteins, and sugar, all expressed in mg/g of dry tissue, and the sugar/amino acid ratio) measured in primitive plants, and the developmental performances of two generalist herbivores. The R^2 coefficients were calculated with the least square method.

why herbivores are unable to successfully develop. As some proteins are involved in defense mechanisms, Markham et al. (2006) have suggested that their overall amounts may hardly be considered as a proper proxy for the effective nutritive quality of the plants. Nevertheless, as defensive proteins and many secondary metabolites in plants can be potent at very low levels (Felton, 1996; Machado et al., 2015), an overall protein content should still be a relevant estimation for dietary quality. When focusing on soluble proteins and digestible carbohydrates in seven Solanaceae and Martyniaceae species eaten by *Manduca sexta* moths (Sphingidae), Wilson et al. (2019) measured levels from 6.9 ±5 to 15.6 ±9.7% for soluble proteins. Despite different analytical procedures, the protein contents in our study, ranging from 1% to 12%, seem to show acceptable contents, and predicting that a major part of these ones can undoubtedly be considered as nutritive, including some initially involved in plant defenses but which could be overcome by herbivores (Bernays & Chapman, 1994, 2000). Based on a cautious interpretation of our results, this study confirms that early land plants offer appropriate global protein amounts, which contribute to dismiss the "lack of nutrients" hypothesis tested. As for most of the plants eaten by herbivores, the different functions of proteins in early land plants remain to be disentangled in order to know exactly which are nutritious.

The levels of free or stored sugars measured in the plants cannot explain the poor performance of polyphagous herbivores either. Half of the moss leaves contained sugar levels that were similar to those found in maize. By contrast, all the pteridophytic plants had significantly lower sugar quantities than maize (Figure 6). Just as for amino acids, overall sugar profiles were found to be taxon-dependent, with bryophytic patterns distinctly separated from both ferns and maize (Figure 7). We found no correlation between plant sugar levels and herbivore growth or survival (Figure 9).

In addition to their energetic roles in plant metabolism, sugars are strongly involved in osmoregulation, especially in mosses, which show exceptional capacities to overcome drought (Bewley, 1979). Sucrose, which occurs in higher levels in mosses than in vascular plants, is particularly important as an osmotic regulator (Proctor, 2000; Smirnoff, 1992), and then as a protective agent against abiotic stress. Frequent desiccation of mosses might be a natural way to consistently challenge the herbivores, as it is difficult to specialize in a continuously changing resource. If so, sugars can be considered as both a nutritive resource and a protective agent in mosses. In the current study, however, this duality was not measurable, as only fresh and well-hydrated plant material was used either for feeding or for chemical analyses.

By themselves, the amounts of amino acids or sugars in plants might not be entirely informative when determining their suitability for herbivores. We therefore also calculated sugar/amino acid ratios for each plant species (Figure 8). Regarding all the plants tested, these ratios cannot be linked as a factor explaining the reduced survival and mass lost in herbivores (Figure 9).

In this study, the notion that mosses and ferns offer reduced nutritive contents for herbivores is strongly refuted. Nevertheless, and paradoxically, our observations reinforce the view that generalist herbivores perform poorly when feeding on early land plants. Our results, as those of Haines and Renwick (2009), may potentially depend on the species of plants and herbivores chosen. Aiming to test the early plants' low nutrient hypothesis, a clear focus on bryophytes and pteridophytes was dictated in this study, without ignoring that the addition of more angio- and gymnosperm species, as well as other herbivore types, would certainly contribute to have broader conclusions.

An alternative explanation for why current herbivores struggle to survive on these plants could be due to specific defense traits. Physical and chemical defenses must have evolved in these plants during their interactions with early herbivores. Physical defenses in primitive plants are considered weak, especially in mosses, which lack hard sclerenchyma tissues (Matsunaga et al., 2004). Current literature on mosses and ferns provides little information on physical traits that serve in a defensive context. Only trichomes of the aquatic fern *S. natans* have been well-studied (Barthlott et al., 2009). However, these trichomes seem to be used more as flotation buoys than protective structures, as they appear not to deter aquatic herbivores that occasionally feed on these plants and others of the same genus (Tewari & Johnson, 2011). Physical defenses in bryophytes and pteridophytes await further scientific description and functional studies.

On the chemical side, certain defenses must have been involved in the early interactions between first land plants and newcomer herbivores. For bryophytes, some chemical compounds have been studied, mainly in liverworts and mostly from a medical application perspective. Liverworts are known to contain secondary metabolites in organelles called oil bodies, which were first described by Hübener (1834) but have only recently been studied from a chemical perspective, revealing lipophilic sesqui- and diterpenoids, phenolic compounds, and polyketides (Asakawa & Ludwiczuk, 2017; He et al., 2013). Chemicals produced by other mosses still need to be properly investigated. Above all, further investigations in this field should focus on the role of secondary metabolites in the plants' interactions with herbivores (Markham et al., 2006). Chemical compounds produced and released by pteridophytes have been slightly more studied. For instance, clubmosses (*Lycopodium* sp.) exhibit complex alkaloids (Aver & Trifonov, 1994; Kitajima & Takayama, 2011), and many chemical compounds produced by bracken ferns (i.e., *Pteridium* spp.) have been described (Jones & Firn, 1978; Schreiner et al., 1984). Cooper-Driver (1978) studied insect-fern associations and the role of secondary metabolites in the defense of bracken ferns against herbivory. It appears that they indeed contain defense compounds that have an impact on pathogens and herbivores (Agarwal et al., 2018).

If less relevant for pteridophytes, mosses frequently show small vegetative structures limiting their overall biomass in terrestrial ecosystems. De facto, these features could exclude many herbivores to exploit these plants, including large insects unable to finalize their development on such limited resources, and contributing to limit herbivores' diversity on bryophytes. However, smaller invertebrates (e.g., Tardigrada) have been shown to perfectly develop on mosses. Possible damages in bryophytes by tiny herbivores would definitely require more attention.

Overall, it appears from our results that the attested dislike of herbivores for bryophytes and pteridophytes is not due to a lack of nutritional value but rather to not yet understood physical and chemical defenses, supporting the same conclusions as Haines and Renwick (2009). Therefore, follow-up investigations on possible defensive mechanisms are needed. They may finally provide an explanation for the paradox of early land plants as a plentiful but neglected resource.

5 | CONCLUSION

In controlled feeding experiments, it is shown that two polyphagous herbivores cannot properly develop and survive on 15 representative bryophytic and pteridophytic species. These findings confirm the standing notion that nonspecialized herbivores perform poorly and avoid feeding on such plants. The analyses of nutrient contents, however, suggest that the normally assumed explanation of poor nutritional value of bryophytes and pteridophytes is not what is responsible for the failure of the herbivores to develop on these plants. In terms of total proteins, amino acids, and sugar contents, the plants offer suitable amounts and concentrations, comparable to maize plants. The levels of these primary metabolites found in fresh leaves should be sufficient for the proper development and survival of both caterpillars and snails. Past assumptions that the bryophytes and pteridophytes' tissues are of poor nutrient quality should therefore be considered incorrect. Yet, as many early land plants are indeed unsuitable resources for herbivores, other factors must be involved. We predict that defensive mechanisms in bryophytes and pteridophytes are likely responsible for the poor performance and avoidance by herbivores.

AUTHOR CONTRIBUTIONS

Audrey Duhin: Conceptualization (supporting); data curation (equal); investigation (lead); writing – original draft (equal). **Ricardo A. R. Machado:** Formal analysis (equal); writing – review and editing (equal). **Ted C. J. Turlings:** Conceptualization (equal); supervision (equal); writing – review and editing (equal). **Gregory Röder:** Conceptualization (equal); data curation (equal); formal analysis (equal); funding acquisition (lead); investigation (supporting); methodology (supporting); project administration (lead); supervision (lead); writing – original draft (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

All data supporting the manuscript are publicly available from the Dryad Digital Repository (https://doi.org/10.5061/dryad.4xgxd25dq).

ORCID

Ricardo A. R. Machado https://orcid.org/0000-0002-7624-1105
Ted C. J. Turlings https://orcid.org/0000-0002-8315-785X
Gregory Röder https://orcid.org/0000-0002-7110-294X

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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3.2 Supplementary material

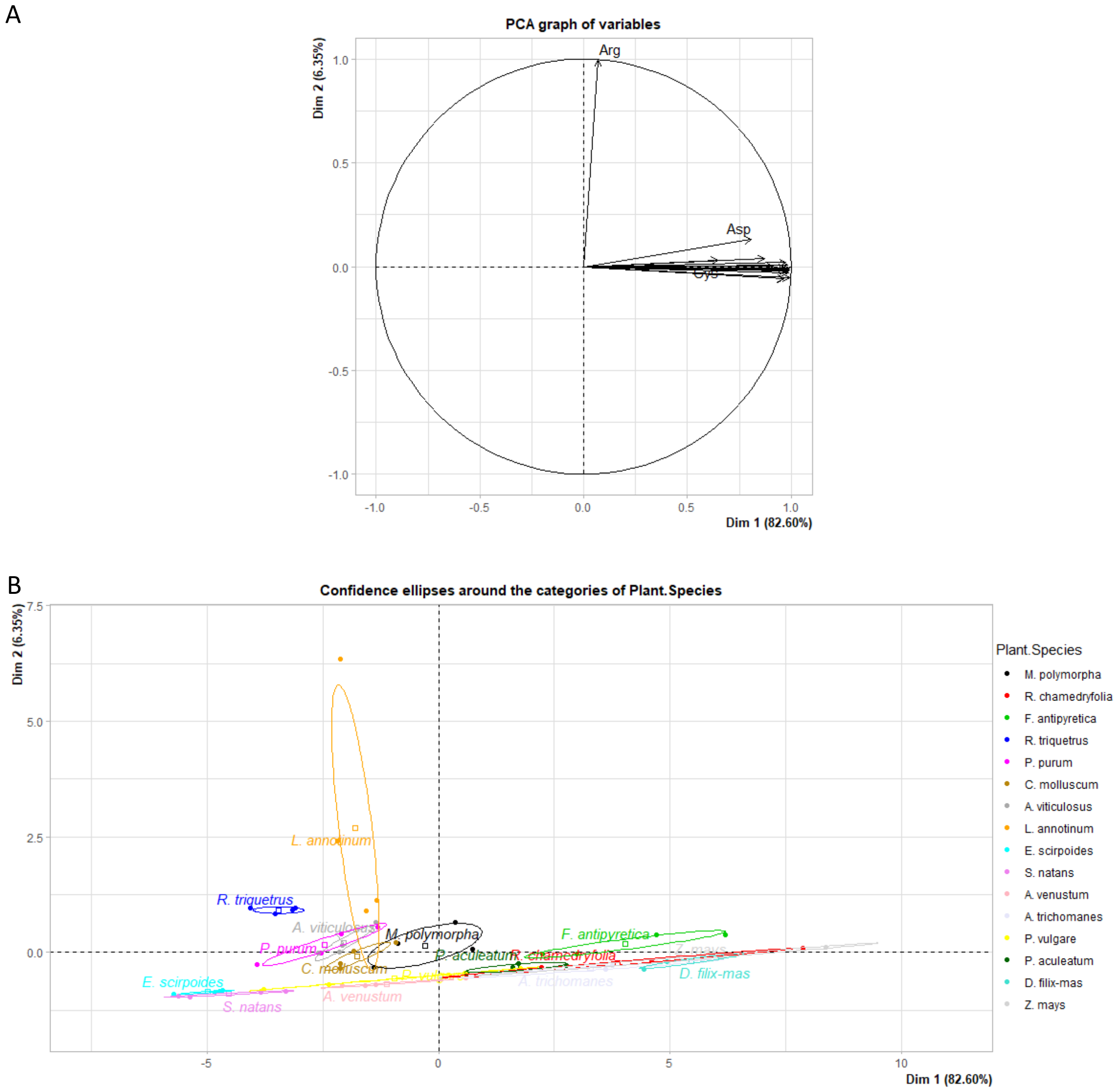

Figure 1: Principal Component Analysis (PCA) based on the 16 amino acids quantified (n=4) in 15 early land plant (7 mosses and 8 ferns) and one angiosperm (maize) species, showing (A) the vectors formed by respective variables (amino acids) in the PCA projection and (B) the confident ellipses (95%) formed around the plant species.

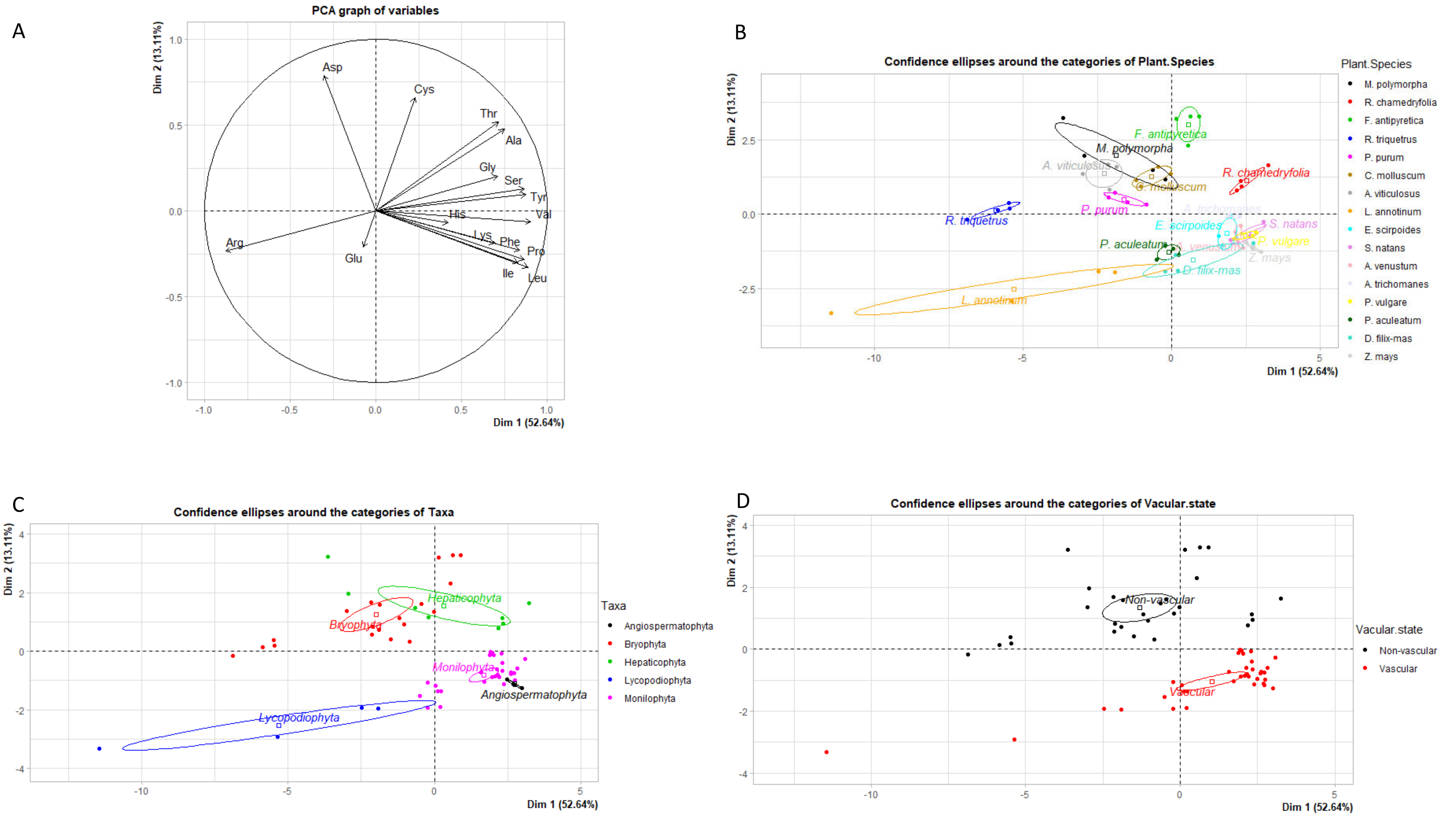

Figure 2: PCA based on the relative amounts of 16 amino acids quantified (n=4) in 15 primitive plants, including 7 bryophytes, 8 pteridophytes and one angiosperm (maize) showing (A) the vectors formed by the originate variables in the PCA projection; the confident ellipses (95%) formed around (B) the plant species, (C) plant taxa and (D) plant vascular-state group.

Table 1: PerMANOVA and pairwise comparisons on amino acids contents results. The post-perMANOVA performed (1) is reported in the bottom left triangle on rough data, and (2) in the upper right triangle on relative amount. The perMANOVA was performed with adonis function (999 permutations, Bray-Curtis distance method). The non-significantly different pair ($p > 0.05$) are written in white in gray cells.

Species	*M. polymorpha*	*R. chamedryfolia*	*F. antipyretica*	*R. triquetrus*	*P. purum*	*C. molluscum*	*A. viticulosus*	*L. annotinum*	*E. scirpoides*	*S. natans*	*A. venustum*	*A. trichomanes*	*P. vulgare*	*P. aculeatum*	*D. filix-mas*	*Z. mays*
M. polymorpha		0.018	0.025	0.012	0.006	0.018	0.028	0.001	0.026	0.026	0.019	0.015	0.025	0.017	0.011	0.024
R. chamedryfolia	0.034		0.031	0.024	0.017	0.031	0.022	0.005	0.032	0.032	0.03	0.026	0.022	0.02	0.015	0.031
F. antipyretica	0.001	0.659		0.006	0.001	0.017	0.005	0.003	0.009	0.004	0.008	0.016	0.02	0.001	0.001	0.003
R. triquetrus	0.015	0.001	0.008		0.001	0.001	0.008	0.003	0.022	0.006	0.001	0.001	0.007	0.004	0.001	0.001
P. purum	0.014	0.001	0.028	0.095		0.023	0.009	0.001	0.032	0.017	0.022	0.018	0.015	0.018	0.023	0.022
C. molluscum	0.019	0.001	0.001	0.001	0.451		0.016	0.01	0.009	0.007	0.003	0.001	0.009	0.001	0.004	0.001
A. viticulosus	0.055	0.001	0.023	0.001	0.314	0.118		0.001	0.024	0.027	0.018	0.019	0.036	0.029	0.019	0.03
L. annotinum	0.001	0.001	0.001	0.001	0.001	0.014	0.001		0.017	0.02	0.024	0.028	0.02	0.022	0.025	0.02
E. scirpoides	0.018	0.001	0.006	0.02	0.028	0.001	0.014	0.017		0.006	0.012	0.001	0.005	0.012	0.001	0.012
S. natans	0.016	0.001	0.006	0.007	0.048	0.003	0.011	0.014	0.519		0.013	0.014	0.001	0.001	0.085	0.016
A. venustum	0.039	0.001	0.006	0.001	0.037	0.029	0.001	0.034	0.035	0.022		0.032	0.806	0.005	0.006	0.024
A. trichomanes	0.037	0.607	0.303	0.005	0.001	0.012	0.008	0.021	0.02	0.014	0.01		0.013	0.027	0.026	0.017
P. vulgare	0.232	0.027	0.001	0.001	0.4	0.478	0.385	0.024	0.023	0.037	0.004	0.066		0.036	0.003	0.001
P. aculeatum	0.001	0.167	0.001	0.001	0.001	0.017	0.001	0.017	0.023	0.019	0.001	0.272	0.009		0.006	0.001
D. filix-mas	0.011	0.19	0.05	0.006	0.001	0.01	0.002	0.033	0.012	0.02	0.099	0.014	0.016	0.003		0.009
Z. mays	0.062	0.631	0.459	0.007	0.004	0.009	0.004	0.015	0.021	0.009	0.027	0.362	0.07	0.127	0.429	

4 Chapter 2: Physical traits of early land plants do not explain the poor developmental performance of a generalist herbivore

Audrey DUHIN[1], Ted C.J. TURLINGS[1], Gregory RÖDER[1]

[1]FARCE laboratory, Institute of Biology, University of Neuchâtel (Switzerland)

4.1 Abstract

Plants and herbivores are engaged in a co-evolutionary arms race, which has resulted in a variety of different types of defensive strategies that enable plants to overcome herbivore attacks. These plant defensive traits include relatively large physical structures like spines and trichomes, but also microscopic ones, like cell wall sclerification and silica incrustations. To some extent, low water content can also serve as a physical defense, decreasing significantly the ingestibility and consumptive value of plant tissues for herbivores. To investigate the importance of such traits in early land plants, like mosses and ferns, we tested how an artificial diet mixed with plant tissue affects the developmental performance of caterpillars of the generalist herbivore *Spodoptera exigua*. In parallel, we used a comparative approach to specifically investigate the effects of three selected physical traits: the presence of trichomes, the content of silica and water concentration, using 15 bryophytic and pteridophytic plants species, as well as two angiosperm species. Our results showed poor developmental performances of the caterpillars on the terrestrial ferns. Regarding the potential physical defenses, no trichome were recorded, except on an aquatic fern species which uses them for floatation. Furthermore, no significant difference between the silica content among the tested plant species and no correlation between this trait and the developmental performances of the herbivores were found. Yet, water concentrations of the plant tissues were significantly lower in terrestrial ferns, compared to all the other plants tested. However, as the water content in artificial diet that was used to feed the caterpillars was similar for all treatments, this cannot explain the poor performances observed on diets with fern powder. Our results show that trichomes, silica and water content are not responsible for the poor developmental performance and survival of the generalist herbivore *S. exigua* when fed on early land plants. Our observations predict that other types of defenses limited presence of herbivores on such plants.

4.2 Introduction

Plants and their associated herbivores are mainly involved in antagonist relationships. The plants have adapted to tolerate an attack by herbivores and/or employ specific defense traits (Strauss and Agrawal, 1999). Any of these defense traits can be countered by a corresponding adaptation by targeted herbivores, leading to an arms race against each other (Ehrlich and Raven, 1964). In this context, plants have evolved ways to avoid or diminish the pressure they endure from the herbivores, by physical or chemical defenses which can be constitutive or induced specifically after attack (Zangerl and Rutledge, 1996).

4.2.1 Physical defenses

Thorns, spines, and prickles, which are derived from different plant structures (*i.e.* stems or auxiliary meristems, modified leaves, epidermal or cortical tissues), are macroscopic physical defenses (Armani et al., 2019). Trichomes can also be physical barriers and can be classified in two main categories: 1) non-vascular trichomes shared by bryophytes, pteridophytes, gymnosperms and angiosperms, and 2) glandular secreting trichomes (GSTs), that require a vascular system (Wagner et al., 2004). The earliest trichomes record comes from an extinct, very basal Spermatophytina taxon, belonging to the seed ferns (Pteridospermae), which shows touch-sensitive glandular trichomes. Supposedly, these were able to release sticky exudates, equivalent to those of some present-day angiosperms, and were probably used to reduce herbivory. This trait might be shared with a common ancestor of all the seed plants (Krings et al., 2002). Extrafloral nectaries are another type of structure found in some ferns (bracken) only, that are supposed to act as indirect defenses by attracting ants and ensure a protection against herbivores (Heads and Lawton, 1984).

Microscopic defense structures containing silica (*i.e.* silicon dioxide) also plays a physical defensive role (Epstein, 1994, Reynolds et al., 2009) as do macromolecules contained in the cell wall (*i.e.* cellulose, lignin), and prevent herbivores from easily consuming plant tissues (Hanley et al., 2007, Freeman and Beattie, 2008). Moreover, there is an important role played by silicon in growth, mineral nutrition, mechanical strength, as well as resistance to fungal and bacterial pathogens, herbivory, adverse chemical conditions of the environment, and leaf longevity (Epstein, 1994, Fauteux et al., 2005, Wang et al., 2017, Cooke and Leishman, 2011). This element is far less studied in early land plants, like mosses and ferns, than in seed plants. In a meta-analysis on plant silica, Hodson et al. (2005) recorded 68 species from the first group only, against 667 seed plant species that were considered. Within the bryophytes and pteridophytes, most relevant studies about the mechanical support provided by silicon, as well as resistance against fungal infection, were carried out with horsetails (Equisetaceae) (Yamanaka et al., 2012, Guerriero et al., 2018).

4.2.2 Water content

Early land plants like bryophytes and pteridophytes have a high dependency on water as both rely on this molecule for their sexual reproduction and bryophytic gametophytes do not show any vascular structures unlike higher plants (Renzaglia et al., 2000). Mosses are the first plants that colonized terrestrial habitat (Nickrent et al., 2000) so they had to develop desiccation tolerance strategies (Oliver et al., 2005, Proctor and Pence, 2002). For the early vascular plants that are pteridophytes, gametophytes are quite similar to bryophytic ones regarding desiccation tolerance (Watkins Jr et al., 2007), whereas the fern sporophyte has been demonstrated to efficiently transport water (Watkins Jr et al., 2010, Pittermann et al., 2013). Juneau and Tarasoff (2012a) even showed that two fern species contain the same average water contents as many seed plants.

There is a traditional view that water-stressed plants are more susceptible to insect herbivory (White, 1984), but this assumption is not entirely supported by evidences (Huberty and Denno, 2004). To some extent, low water content is considered as a physical trait influencing the consumption of plant tissues by herbivores. Indeed, there is a positive correlation between water content and herbivory in higher plants (Poorter et al., 2004), and the palatability of the leaves for insect herbivores is also positively correlated with water content (Schädler et al., 2003). Insects are also able to influence the plant host quality in term of leaf toughness and tannin concentration, but not in water content (Sagers, 1992).

4.2.3 Other defenses

The other main types of defenses involved against herbivores rely on secondary metabolites. These chemical defenses have been more investigated in bryophytes and pteridophytes than the physical defenses. We can for instance find several classes of secondary metabolites in mosses, especially in liverworts which can contain oxylipins (Croisier et al., 2010, Ponce de Leon et al., 2015, Rempt and Pohnert, 2010), terpenes, aromatics (He et al., 2013), and sesquiterpene lactones (Asakawa and Takemoto, 1979, Knoche et al., 1969). In the group of the bryophytes, there are species containing phenols (Davidson et al., 1990, Davidson et al., 1989) that are repellent to gastropods and isopods (Glime, 2006), or in the aquatic *Fontinalis* sp. fatty acids that repel insects (Parker et al., 2007). When looking at early vascular plants, clubmosses are known for their alkaloids (Aver and Trifonov, 1994, Kitajima and Takayama, 2011) and ferns contain phenols (Bohm, 1968, Bohm and Tryon, 1967), as well as flavonoids (Star and Mabry, 1971), whereas some species produce cyanogenic glucosides which have a negative effect on insect development (Schreiner et al., 1984). Primary metabolites like low protein content can also be used as defenses (Markham et al., 2006). It has also been hypothesized that the low herbivory observed on both mosses and ferns may be due to the poor nutritional value offered by these plants to herbivores (Hendrix and Marquis, 1983, Herbert and Prins, 1982).

In a previous study, we showed that two distinct generalist herbivores, a caterpillar and a snail, that were fed with fresh leaves of 15 primitive plant species suffered poor developmental performances and survival, despite appropriate nutritional contents provided by the mosses and the ferns considered. The poor nutrient hypothesis in these early land plants was strongly refuted (Duhin et al., 2022). As a follow-up study, we investigate here the possible physical defenses deterring herbivory in the same selection of 15 bryophytes and pteridophytes. We tested the ability of a generalist herbivore caterpillar (*Spodoptera exigua*) to survive and develop on a mixture of ground tissue from early land plants and a commercial artificial diet designed for lepidopteran rearing, thereby excluding the effects of macrophysical defenses. In parallel, we measured physical traits that could be involved in plant defenses, namely silica and water contents, and assessed their possible roles in the performances of the herbivore.

4.3 Material and methods

4.3.1 Plants

Wild plants were collected in and near of the canton of Neuchâtel (Switzerland). If not available in their natural habitat, they were grown in the botanic garden (Jardin Botanique, Neuchâtel) or purchased in a specialized garden center (Flower Market Dietrich, GmbH). Two weeks before each experiment, all plants were acclimatized at the University of Neuchâtel in a plant growth chamber (CLF Plant Climatics, Germany) with the following conditions: 13-15°C; 70% RH; day/night photoperiod of 12:12hrs (light:dark). All plant species (with their respective three letters codes) used for feeding experiments and chemical analyses belong to the following three groups: 1) bryophytes with two liverworts species (*Marchantia polymorpha* (Mpo) and *Riccardia chamedryfolia* (Rch)) and five moss species (*Fontinalis antipyretica* (Fan), *Rhytidiadelphus triquetrus* (Rtr), *Pseudocleropodium purum* (Ppu), *Ctenidium molluscum* (Cmo), and *Anomodon viticulosus* (Avi)); 2) pteridophytes, including one clubmoss species (*Lycopodium annotium* (Lan)), seven monilophytes (one horsetail: *Equisetum scirpoides* (Esc); and six ferns: *Salvinia natans* (Sna), *Adiantum venustum* (Ave), *Asplenium trichomanes* (Atr), *Polypodium vulgare* (Pvu), *Polystichum aculeatum* (Pac) and *Dryopteris filix-mas* (Dfi)); and 3) two angiosperms, *Zea mays* var. Delprim (Zma) and *Lactuca sativa* var. capitate (Lsa), which were used for comparison. When harvested, plant leaves were instantaneously frozen in liquid nitrogen. They were ground under liquid nitrogen, using a mortar and a pestle, to obtain a powder for each species that was weighed and dried to measure the water concentration. Silica content was determined using the dried ground plant tissues. The powders were also used during the bioassays by mixing with the artificial diet to create a series of different mixed diets (semi-artificial diets hereafter).

4.3.2 Herbivore

The generalist herbivore used in this study was second instar caterpillar of the beet armyworm *Spodoptera exigua* (Lepidoptera: Noctuidae) reared on artificial diet prior to feeding experiments (Beet Armyworm diet F9219B, Frontier Agricultural Sciences, Newark, DE, USA) at the University of Neuchâtel. Eggs for the rearing colony were initially obtained from Entocare (Wageningen, The Netherlands).

4.3.3 Bioassays with semi-artificial diets

Of each of the 15 primitive species and the two angiosperm species, 18±3 g of dry plant powder was mixed within 200 mL of artificial diet (Frontier soy gem mix), poured in plastic box to cool down and stored at -20°C before experiments. Pure artificial diet was used as the control treatment. *S. exigua* second instar larvae (n=16 caterpillars per treatment) from our rearing were then individually placed into cells (4 x 4 cm surface 2.5 cm high) of plastic trays closed thanks to plastic perforated lids (RT32W and RTCV4 respectively, Frontier Agricultural Sciences, Newark, DE, USA), and provided *ad libitum* with one of the diet treatments. The caterpillars were weighed every three days and the diet renewed. The larval weights at day 12 received particular attention (two third of the development), representing an interesting approximation for the future development success of *S. exigua*. If relevant, both pupation and adult emergence days were recorded.

4.3.4 Physical traits

4.3.4.1 Assessment of physical defensive structures

Under a binocular loupe, both spines and trichomes' presence was investigated by meticulous examination of several individuals of every fern species. The mosses do not possess such or equivalent structures. Close-up pictures were taken when required. Literature review suggests that none of these species show any macroscopic defensive structures, excepted for the well-studied trichomes of the aquatic fern *Salvinia natans* (Salviniaceae) (Barthlott et al., 2009).

4.3.4.2 Silica quantifications

Silica content in leaves was quantified in the Laboratory of Functional Ecology at University of Neuchâtel. The method was developed by Hallmark et al. (1983) and slightly adapted according to Callis-Duehl et al. (2017). The material was ground under liquid nitrogen and freeze-dried during 24h at -50°C (freeze-dryer Labconco FreeZone 2.5, LABCONCO, Kansas City, MO, USA; coupled with a pump RV8, Edwards, Burgess Hill, West Sussex, UK). Approximately 50 mg of dry material was then used for the analyses. The digestion process started with 1-octanol and hydrogen peroxide (H_2O_2) heated at 90°C during 30 minutes. After the cooling to room temperature, sodium hydroxide (NaOH) is added and the samples are again heated at 90°C, for 4 hours. Afterwards, ammonium fluoride (NH_4F) is added, the sample diluted with water and aliquots made. Those aliquots were diluted with water and mixed with acetic acid and ammonium molybdate. After 10 minutes wait, tartaric acid and 1-amino-2-naphthol-4-sulfonic acid coloration solution were added and the samples were left in the dark for 30 minutes before an absorbance measure at 650nm was carried out, using a Multiskan GO spectrophotometer (ThermoFisher Scientific, Vartaa, Finland). The results were then converted to silica contents based on a calibration curve made with solutions containing different silicon dioxide (SiO_2) concentrations. Those quantifications were done with all the studied plants as well as with the pure control artificial diet used as a control (supp. Fig. 1).

4.3.5 Water contents

The water content of the plants was assessed by weighing aliquots of ground plant leaves using a balance (Mettler-Toledo, model AC100), before and after freeze-drying of the sample for 24h at -50°C (Labconco FreeZone 2.5, LABCONCO, Kansas City, MO).

4.3.6 Statistical analysis

Statistical analyses were carried out in R studio (R version 3.4.3). To assess the effect of the different diets on caterpillar development (*i.e.* mass at several time points), and both silica and water contents in plants, non-parametric Kruskall-Wallis tests were performed as raw data do not meet assumption of homoscedasticity and normal distribution of residues, even when tested after transformations. When a significant difference was found between treatments, those tests were followed by post-hoc analysis (using the criterium Fisher's least significant difference, alpha = 0.05 and Bonferroni’s p-values adjustment). Additionally, a linear regression for the caterpillar weights at day 12 and silica or water contents in different plants was carried out.

4.4 Results

4.4.1 Bioassays with semi-artificial diets

Regarding the mass gain at day 12, and compared with maize, lettuce and pure artificial diet, *Spodoptera exigua* caterpillars fed on different mixed diets containing early land plant powder were found to be able to develop properly on bryophyte tissues and on the aquatic fern *Salvinia natans* (Fig. 1A). In contrast, their development was significantly worse in terms of mass on all the other pteridophyte species like clubmoss, horsetail and terrestrial ferns (*Lycopodium annotinum*, *Equisetum scirpoides*, *Adiantum venustum*, *Asplenium trichomanes*, *Polypodium vulgare*, *Polystichum aculeatum* and *Dryopteris fillix-mas* (Fig. 1A)). Pupation and adult emergence rates followed the same pattern as the mass at day 12, with caterpillars showing a low mass at that time of their development being unable to reach neither pupation nor adult stages (Fig. 1B and 1C, respectively).

4.4.2 Silica content

Silica content values showed no significant difference between the studied plants tested (Kruskal-Wallis test, Chi^2 = 16.496, df = 16, p-value = 0.419). We observed high intraspecific variation within most of the species, with silica content (% of dry material) ranging from 0.13 to 0.72 % in bryophytes and pteridophytes, whereas it was more consistent within the lettuce and the maize plants (0.16 and 0.24 % respectively) (Fig. 2). No correlation between caterpillar developmental performance and silica content in plants can be observed (Fig. 4).

4.4.3 Water content

The water contents measured in most of the fresh fern species were significantly lower compared to mosses and the two seed plants, except for the aquatic fern *Salvinia natans* (Kruskal-Wallis test, Chi^2 = 93.990, df = 16, p-value < 0.001, post-hoc tests, alpha = 0.05) (Fig. 3). A correlation was found between the caterpillar mass after 12 days (Fig. 4B) and the fresh water contents, even though these were not measured directly in the semi-artificial diets used in the feeding bioassays.

4.5 Figures

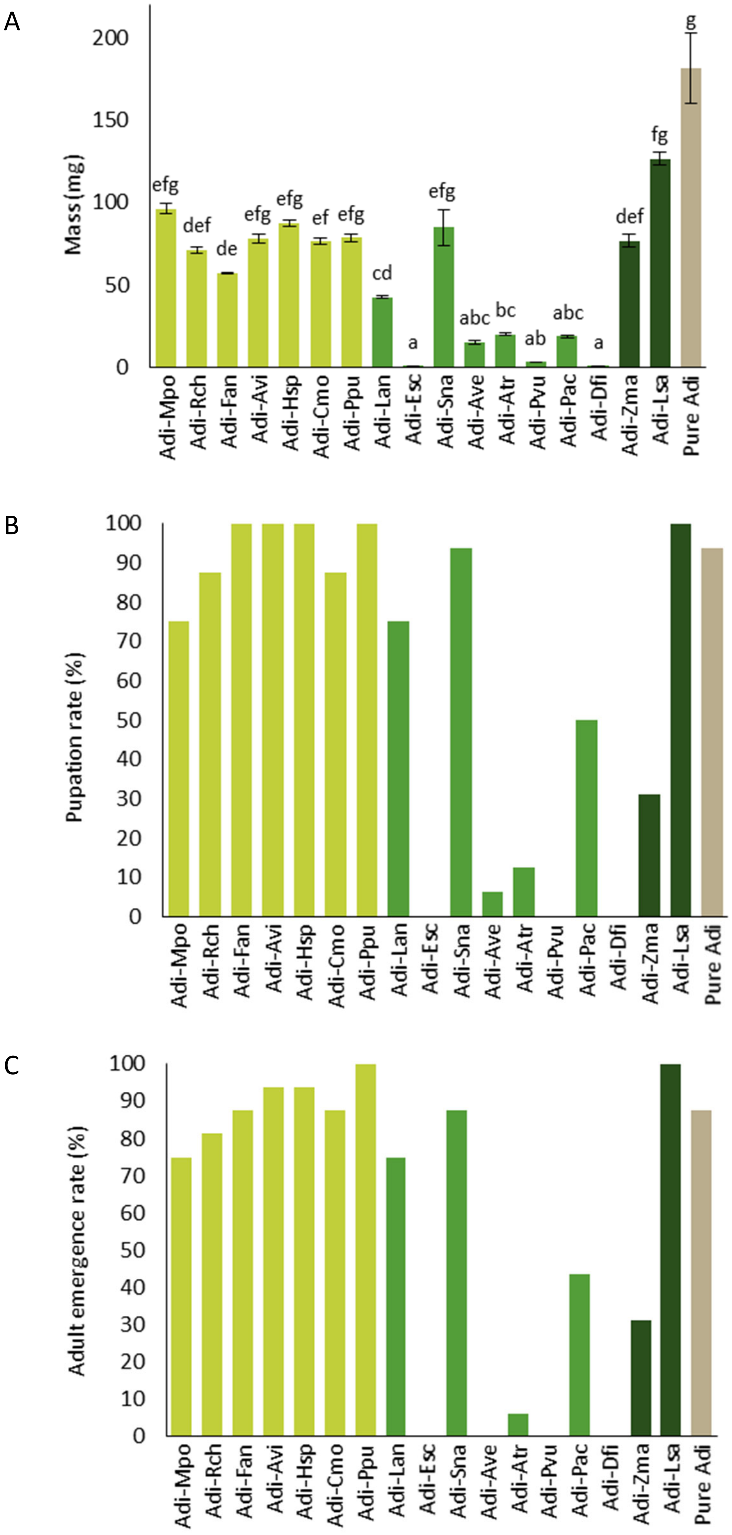

Figure 1: *Spodoptera exigua* developmental performances when fed on a pure artificial (pure Adi, borwn bar) or 17 semi-artificial diets containing ground leaf material from 7 bryophytes (light green), 8 pteridophytes (green) and 2 angiosperms (dark green): (A) Mass of the caterpillars at day 12 (in mg, mean ± SE) with the different letters on top of bars represent significant difference (Kruskal-Wallis & post-hoc analysis, $p < 0.05$); (B) Rate of pupation (%); and (C) rate of adult emergence (%).

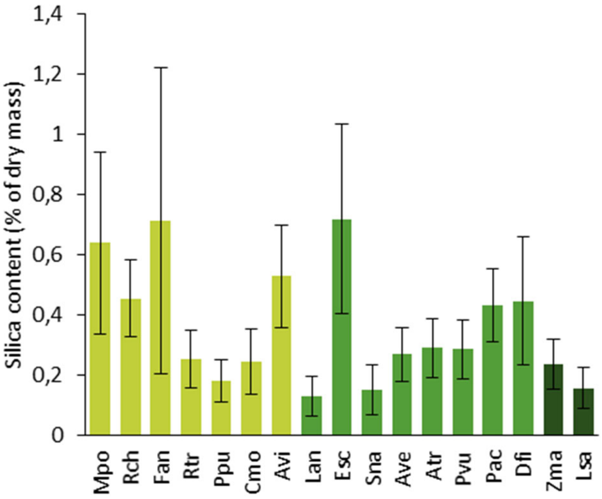

Figure 2: Silica content (% of the dry mass, mean +/- SE) in 7 bryophytes (light green), 8 pteridophytes (green) and 2 angiosperms (dark green) (Kruskal-Wallis test, Chi^2 = 16.496, df = 16, p-value = 0.419).

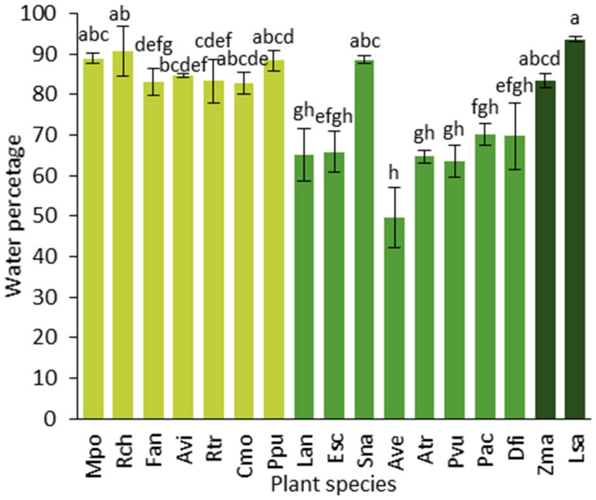

Figure 3: Water content (% of the fresh weight, mean +/- SE) in 7 bryophytes (light green), 8 pteridophytes (green) and 2 angiosperms (dark green) (Kruskal-Wallis test, Chi^2 = 93.990, df = 16, p-value < 0.001). Letters above the bars indicate significant differences (post-hoc test, alpha = 0.05, p-adjust method = Bonferroni).

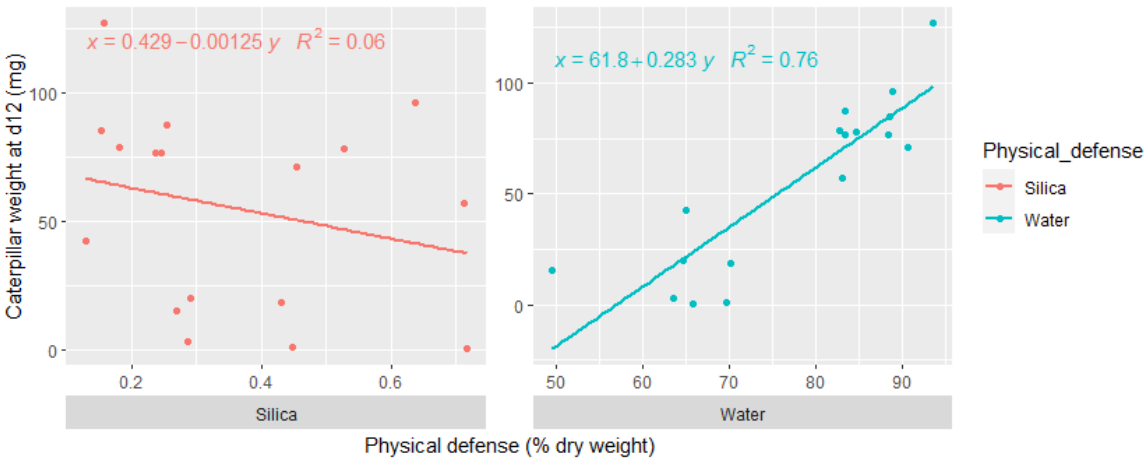

Figure 4: Linear regression between A) silica contents (% dry weight)) and B) water contents (% fresh weight) measured in 15 moss and fern species and the weight of *Spodoptera exigua* herbivores at day 12 of their development. No correlation can be found for the silica contents (A), whereas fresh water contents (B) were measured but not directly tested in the semi-artificial diets used in the feeding bioassays.

4.6 Discussion

4.6.1 Developmental performances of the herbivores on early land plants

Compared with the mosses, the two angiosperms, or the pure artificial diet, *S. exigua* development was significantly poorer when fed on the diets containing the terrestrial ferns, as measured in body mass, as well as pupation and adult emergence rate (Fig. 1). Poor developmental performance of herbivores appears to be common on early vascular plants (Cooper-Driver, 1978, Agarwal et al., 2018). The relatively good performances of the caterpillars that were fed on different moss diets contradict both our initial hypothesis of a likely unsuitability of bryophytes, and avoidance and/or bad performance observed on these plants in previous studies (Glime, 2006, Gerson, 1969, Haines and Renwick, 2009). Usually, mosses are only consumed by herbivores in harsh environments or if nothing else is available (Prins, 1982, Smith et al., 2001, Crafford and Chown, 1991). There is also a low nutritive value and low digestibility theory for mosses that may explain the rare occurrence of herbivory on these plants (Haines and Renwick, 2009). In a previous study, we also observed bad performances of *S. exigua* caterpillars, as well as a herbivorous snail (*Cochlicella barbara*), when fed with fresh leaves of the same plant species that were tested here. From the analyses performed for this previous study we can conclude that nutrient levels in bryophytes and pteridophytes (proteins, amino acids, sugars) cannot account for poor herbivores performances, as all the plants had similar amounts of nutrients as the angiosperm control (*Zea mays*, variety Delprim). This is what promoted the current study and the prediction that physical and/or chemical defenses in early land plants prevent herbivores from feeding on them(Duhin et al., 2022). Wishing to disentangle these types of defenses, the current study was designed both to assess the deterring effect of possible physical defenses and to test a representative panel of known physical defenses present in early land plants. As mentioned, bryophytes seem to lack physical defenses, so we were not expecting to find a reduction of the negative effect of bryophytic tissues as a diet on the caterpillars' development as seen in Duhin et al. (2022). In this study, however, ground bryophytes mixed in artificial diet appeared to be perfectly suitable for the caterpillars, and there was no obvious performance correlation with silica or water content. However, it might be that some potent constitutive moss chemical defenses may have been diluted and/or altered through the preparation process of the semi-artificial diets, reducing the usual suppressive effect on herbivory. For the pteridophyte species, this reduction in unpalatability was not observed. This is surprising because the semi-diets were processed in the exact same way as the bryophytic and pteridophytic diets, and implies that the chemical defenses differ markedly between these two early land plant groups.

4.6.2 Absence of macrostructures

We found no major macroscopic physical defense in the early terrestrial plants. Only one species exhibits trichomes, the aquatic fern *Salvinia natans*, which uses them to trap air in the hairy structure of these trichomes that make them flotation features, ensuring buoyancy for the plant (called the *Salvinia*-effect, (Barthlott et al., 2017). Yet, these abundant and imposing trichomes do not prevent the consumption and dissemination of *S. natans* by vertebrate herbivores like ducks (Lovas-Kiss et al., 2018). Regarding glandular trichomes, their presence is rare in ferns and unreported in mosses (Krings et al., 2002).

For the bioassays, we purposely got rid of most of the physical traits and structures that could be used as defense by grinding the leaves, even though we did not specifically investigate microtrichomes that could potentially be on the surface of the fern’s leaves (Rodriguez et al., 2018). These microstructural trichomes are not considered as a physical barrier but rather a means to expose attackers to chemical defenses. The same kind of external secretion is also known from the leaves of certain fern species may that exude chemical compounds, which ones might impact herbivores as well (Wollenweber et al., 1998).

4.6.3 Silica content and its defensive role in early land plants

Silica content was found to be quite variable both intra- and interspecifically in the bryophytes and pteridophytes species studied here (Fig. 2). Silica in early land plants has been, and still is, only sparsely investigated. To our knowledge, only one meta-analysis comparing silica contents in different plant taxa was conducted by Hodson et al. (2005), considering 600 species of angiosperms, 67 gymnosperms, 59 ferns, and only five clubmosses, two horsetails, one moss and one liverwort. In higher plants, silica is globally considered as positively correlated with the defense against herbivorous insects, with measurable effects up to the third trophic level (Reynolds et al., 2009, Kvedaras et al., 2010).

Moreover, silicon metabolism is complex and this element is involved in many physiological processes. We here studied silica content in the context of defense against herbivory, but it has a broader importance in plant physiology including growth, mineral nutrition, mechanical strength, resistance to fungal and bacterial pathogens, adverse chemical conditions of the environment, and leaf longevity (Epstein, 1994, Fauteux et al., 2005, Wang et al., 2017, Cooke and Leishman, 2011). Silica in horsetails has been shown to play a role in the physical support (Yamanaka et al., 2012) and in defense against fungal infections (Guerriero et al., 2018).

In the Poaceae family, silica accumulation has been proven to be inducible by stress such as herbivory by arthropod with the strongest response occurring late after the attack by herbivores (Massey et al., 2007, Reynolds et al., 2016, Johnson et al., 2021). In our study, the linear regression between the performances of the herbivore and the silica concentration in plants seems to show a weak negative correlation, but with a low $R^2 = 0.06$ (Fig 4). It should be noted that we used wild specimens to have that all may have different background life story experiences and have potentially undergone different previous stresses. Such possible differences may still be reflected in the plants’ defense traits even after the two weeks spent in the controlled growth chamber before experiments.

Alternatively, silica can also be present as needles in the plant tissues causing injuries due to abrasive action on the herbivore body, and increasing the likelihood of infections by pathogens (Lev-Yadun and Halpern, 2019). We did not find any evidence for this plant defense mechanism, and there is no record of it in mosses or ferns.

4.6.4 Low water content used as a defensive trait in primitive plants

In this study, we observed that the water contents are comparatively low in pteridophytes (Fig. 3). In tropical environments, it has been demonstrated that ferns exhibit various traits that negatively affect herbivores, including low water content (Farias et al. (2020). Those findings combined with the fact that a high water content is positively correlated with the good performances of herbivores (Poorter et al., 2004, Schädler et al., 2003), can partially explain the poor performance of herbivores on fresh ferns. We were not expecting this pattern to be visible here as we used dry plant mixed with an artificial diet that provided the same amount of water to all the caterpillars, independent of the diet type. Thus, the correlation between water contents in plants and the caterpillar mass gain shown in the figure 4

was not properly tested in our experiments. Yet, the strong correlation suggests that, indirectly, water content has an effect on the ability of plants to resist herbivory.
Drought stress has been proposed to cause a diminution of chemical defense in plants and possibly affects preference in specialized herbivores, which will prefer non-stressed plant with higher chemicals level, whereas a generalist herbivore will choose a less-defended plant (Gutbrodt et al., 2011). Regarding bryophytes and pteridophytes, desiccation mechanisms have not been studied in the context of herbivory. Mosses are known to have high desiccation tolerance (Proctor, 2001, Wood, 2007), with a specific transcriptome associated (Oliver et al., 2004, Gao et al., 2017). In case of pteridophytes, fern spores are known to contain little water, which facilitates airborne dispersal (Gómez-Noguez et al., 2016), whereas other tissues have been shown to tolerate desiccation to a significant extent (Proctor, 2009, López-Pozo et al., 2018, Anderson, 2021).

We can conclude that physical defenses tested in our study do not explain the overall poor performance of *S. exigua* on most of the early land plants. This leads to the hypothesis that chemical defenses are mainly responsible for protection against insect herbivores, as is known for gymnosperm and angiosperm species. Studies on ferns and their chemical defenses are still scarce and the available data are not consistent enough to conclude that the apparent unsuitability of pteridophytes for herbivores is due to chemical compounds alone (Agarwal et al., 2018, Jones and Firn, 1978, Radhika et al., 2012).

4.7 Conclusion

Silica contents in 15 representative species of early land plants is not a physical defense trait responsible for poor developmental performances of the generalist herbivore *Spodoptera exigua*. To determine whether the varying water contents in fresh bryophytes and pteridophytes may play a significant defensive role requires further exhaustive investigations. We found a surprising significant correlation, despite the fact that the semi-artificial diet used throughout the study offered equivalent water levels. Taken together, our findings exclude some defense mechanisms and open the door for follow-up investigations on likely chemical defenses in early land plants, and especially in ferns. Such studies may help to verify a recent hypothesis suggesting an early emergence of chemical defenses once the first plants colonized terrestrial habitats (He et al., 2013, Rozema et al., 2002).

4.8 Acknowledgments

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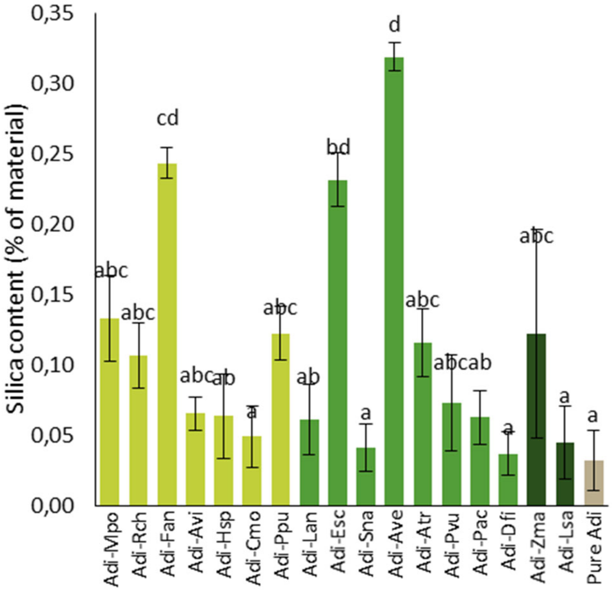
4.10 Supplementary data



Supplementary Figure 1: Silica content of the semi-artificial diet containing primitive plant tissues (mean ± SE). Three measurements were done to ensure that the semi-artificial diet made was containing the same amount of silica than actual plant tissues measured and that this was regularly distributed through the diet. Different letters on top of bars represent significant difference (ANOVA & post-hoc analysis, $p < 0.05$).

5 Chapter 3: Chemical defenses in early vascular plants: the bioactivity of extracts from the clubmoss *Lycopodium annotinum* and fern *Dryopteris filix-mas* against generalist *Spodoptera exigua* caterpillars

Audrey DUHIN[1], Sylvain SUTOUR[2], Gaëtan GLAUSER[2], Ted C. J. TURLINGS[1] and Gregory RÖDER[1]

[1] Laboratory of Fundamental and Applied Research in Chemical Ecology, Institute of Biology, University of Neuchâtel, Switzerland

[2] Neuchâtel Platform in Analytical Chemistry (NPAC), Faculty of Sciences, University of Neuchâtel, Switzerland

5.1 Abstract

The interactions between early land plants and herbivores are considered to be rare, but have been poorly studied. Several decades ago, ecologists hypothesized that both mosses and ferns are of poor nutrient value, which would explain, at least in part the apparent lack of consistent herbivory on these plants. Yet, recent studies carried out on 15 representative bryophytic and pteridophytic species have revealed that they contain appropriate levels of basic nutrients, thus dismissing a "poor nutrient" hypothesis. Physical defenses could also be discarded as being responsible for preventing polyphagous herbivores to feed on these plants.

In the current study, we investigated possible chemical defenses in two early vascular plant species, a clubmoss (*Lycopodium annotinum*) and a fern (*Dryopteris filix-mas*) by testing the effects of extracts from these plants on the generalist herbivore *Spodoptera exigua*. In a first experiment, different solvents (dichloromethane, water and methanol) were used to extract the leaves, and after solvent evaporation the dry extracts were mixed in pure artificial diets. The developmental performance on the caterpillars fed on different diets containing chemical extracts was monitored. In a second step, the most toxic aqueous extract from the fern species was fractionated into 6 fractions, using liquid chromatography, with the aim to isolate and identify the most potent toxin(s). We found that the most harmful fraction for caterpillars contains phloroglucinols. By themselves, these compounds did not have the full toxic or deterring effect on the caterpillars, suggesting that they function in combination, possibly also with other molecules. Our results bring to the fore that chemical defenses in early vascular plants can play a key role against herbivory and indicate an early presence of such defenses in vascular plants.

5.2 Introduction

The early evolution of vascular plants represented an important step in the colonization of terrestrial habitats by plants. The oldest still existing vascular land plants are the pteridophytes, whereas the first univocal record of a plants having a vascular system belongs to the extinct *Cooksonia* genus, which existed about 420 million years ago (Lang, 1937, Edwards et al., 1992). As underlined in Rothwell (1996), the pteridophytes experienced evolutionary success throughout geological time. The pteridophytes (sensu Pryer et al., 2004) are a paraphyletic association of early vascular plants taxa, grouping Lycopodiophyta (contemporary spikemosses, quillworts and clubmosses) and Moniliophyta (horsetails and ferns). Compared to non-vascular bryophytes (mainly mosses), innovations that were developed by pteridophytes are stems with vascular tissue thanks to the use of lignin, and a dominant sporophytic phase as opposed to the gametophyte phase (Renzaglia et al., 2000). Completing the plant evolution, gymno- and angiosperm seed plants evolved later (with *Elkinsia* c.a. 360 M.Y. ago (Rothwell and Scheckler, 1988), relying on new features such as seed, waterless reproduction and flower structures (Friis et al., 2001, Barros et al., 2015).

In parallel, the insects evolved ca. 400 M.Y. ago (Rice et al., 1995, Engel and Grimaldi, 2004). They have coexisted right from their beginnings with pteridophytes, and fossil traces of herbivory have been found, attesting that these early land plants were exploited by early herbivorous insects (Labandeira, 2007, Robledo et al., 2015). Studies on current pteridophytic plants indicate a puzzling low herbivores pressure and diversity (Hendrix, 1980). Because current fern-herbivore interactions have only been scarcely studied, the various explanations concerning the lack of herbivory on early land plants have not yet been properly tested (Hendrix and Marquis, 1983, Haines and Renwick, 2009). One hypothesis has been that pteridophytes have stayed aside of any coevolution with phytophagous insects, unlike angiosperms and entomophily (insect pollination) (Pellmyr, 1992), but this notion was dismissed based on the comparisons of diversification phases of organisms at a geological scale (Labandeira, 2006). This leaves some specific plant traits that may explain the reluctance of herbivores to consume pteridophytes.

For more than half of a century that ecologist have been interested in plant-insect interactions (Ehrlich and Raven, 1964), herbivory on early land plants have been a minor research topic. Some studies, that were mainly carried out in tropical areas, concluded that there are virtually no generalist herbivores feeding on ferns, and they appear to be attacked by only a few specialized on these plants, like certain sawfly species (Hymenoptera: Symphyta) (Lawton, 1976, Lawton, 1982, Ranker, 2016). Trying to explain the apparent avoidance by and bad performances of herbivores on pteridophytes (Agarwal et al., 2018, Cooper-Driver, 1978), several hypotheses have been proposed. Among them, a supposed low nutritive quality of early land plant, but relevant data about nutrients in mosses and ferns are scarce. Although a poor nutrient level has indeed been reported for the aquatic fern *Azolla filiculoides* (Buckingham et al., 1978). However, Duhin et al. (2022) have shown that the nutritive contents in 15 representative bryophytes and pteridophytes show suitable levels and do not correlate with poor developmental performances observed in two generalist herbivores that were fed with fresh plants. Another hypothesis suggests that the amount of vegetative tissue available in bryophyte colonies is very limited and may not be sufficient to feed insects (Noy-Meir, 1975). This concerns bryophytes only, and not ferns. This hypothesis is in contradicted by the fact that small invertebrates, like certain Tardigrada are perfectly adapted to this resource. It has also been suggested that varying water contents may be an obstacle to the consumption of these plants by herbivores (Poorter et al., 2004, Schädler et al., 2003). However, desiccation tolerance is much more important in bryophytes than in vascular pteridophytes, which makes it unlikely that reduced digestibility due to varying water content is a key explanation for the lack of herbivory on these plants (Juneau and Tarasoff, 2012b, Farias et al., 2020).

The interactions between herbivorous insects and seed-producing plants have been extensively studied. In contrast, the relationships between insects and early vascular plants such as clubmosses and ferns are still poorly investigated (Ranker, 2016, Fuentes-Jacques et al., 2021). For gymno- and angiosperms, plant-herbivore theory suggests that both protagonists involved in the interaction suffer antagonistic effects ultimately leading to a co-evolutionary arms race (Ehrlich and Raven, 1964, Thompson, 1994). Surprisingly, very few studies have considered a possible co-evolutionary arms race between herbivores and early vascular plants. In a recent review, however, Fuentes-Jacques et al. (2021) found evidence of coevolutionary patterns between some insects and pteridophytes.

In the pteridophytes, various clubmosses (*Lycopodium* spp.) have been shown to rely on alkaloids for chemical defense against herbivores (Aver and Trifonov, 1994, Kitajima and Takayama, 2011). Ferns also contain secondary metabolites used against herbivores, like phenols (Bohm, 1968, Bohm and Tryon, 1967) and flavonoids (Star and Mabry, 1971). The bracken fern *Pteridium aquilinum* contains cyanogenic glucosides with proven negative effects on insect development (Schreiner et al., 1984).

In the current study we looked for possible chemical defenses in two pteridophytic plants, the clubmoss *Lycopodium annotinum* L. (Lycopodiaceae) and the fern *Dryopteris filix-mas* (L.) Schott (Dryopteridiaceae). The *Lycopodium* genus brings the advantage that it has been well-studied regarding its phytochemistry. They are known to produce alkaloids (Aver and Trifonov, 1994, Kitajima and Takayama, 2011), many of them identified in *L. annotinum* (Orechoff, 1934, Anet and Eves, 1958, Harrison et al., 1961, Koyama et al., 2005, Ishiuchi et al., 2009). However, these likely defensive compounds still need to be tested in a plant-herbivore framework. The terrestrial fern *Dryopteris filix-mas* (L.) Schott (Dryopteridiaceae) has been found to contain secondary metabolites such as phenols or phloroglucinols (Soare et al., 2012, Han et al., 2015) with proven bioactive against insects like caterpillars, aphids, as well as nematodes (Shukla and Tiwari, 2011, Soare et al., 2012, Benoufella-Kitous et al., 2014, Egorova et al., 2021). In a previous study (Duhin et al., 2022), we observed that caterpillars of the beet armyworm (Lepidoptera: Noctuidae, *Spodoptera exigua* Hübner) developed well on semi-artificial diet containing ground leaf tissue of *L. annotinum* clubmoss, whereas same caterpillars suffered on diet containing leaf powder of the fern *D. filix-mas*.

In the current study, we used leaf extracts of the two pteridophytic species to spike artificial diet that was fed to caterpillars of the generalist herbivore *S. exigua*, with the aim to reveal the likely existence of chemical defenses in these early vascular plants against herbivory.

5.3 Material and methods

5.3.1 Plants and herbivore

All the wild clubmoss *Lycopodium annotinum* L. 1753 (Lycopodiaceae) were collected in Noiraigue (Canton of Neuchâtel, Switzerland), and the fern *Dryopteris filix-mas* (L.) Schott 1834 (Dryopteridaceae) collected in Provence (Canton of Vaud, Switzerland). During the week before experiments and chemical analyses, all the plants were kept at the University of Neuchâtel, in a controlled plant growth chamber (CLF Plant Climatics, Germany) under 12:12 hours light:dark photoperiod and a 15 ± 2 °C temperature.

Second instar caterpillars of the beet armyworm *Spodoptera exigua* (Insecta, Lepidoptera: Noctuidae) reared at the University of Neuchâtel used for the feeding bioassays. They were grown and kept under controlled conditions (23±1°C; day/night photoperiod of 12:12hrs) in growth chambers and fed only with lepidopteran artificial diet (Frontiers) since hatching before experiments.

5.3.2 Chemical extractions

The extractions were done on freeze-dried plant tissues that were ground under liquid nitrogen to avoid any chemical degradation. As a first step, three different solvents, namely 1) methanol, 2) dichloromethane, and 3) H_2O + formic acid 0.1%, were used to extract as much different compounds as possible from the plant material. Each extraction was carried out with a 10:1 solvent:plant mass ratio. The extraction was allowed to occur under magnetic agitation overnight and three successive extractions were made on the same plant powder. Each time, the different extracts were filtrated through filter paper and evaporated with a rotavapor, coupled with a freeze-dryer. In the case of the water-based solvent, only the freeze-dryer was used for 72h. The dried residues were then collected and weighed. These were stored at -80°C to be added to the diet used in bioassays or aliquoted to be analyzed. For the second step, after initial feeding bioassays with caterpillars and selection of the most potent solvent extraction, another sample of 225 mg of the aqueous extract of *Dryopteris filix-mas* was fractionated on a silica column (20 g, 63-200 µm, 60 Å) in the six following fractions: F1) eluted with heptane (200 mL), F2) heptane/dichloromethane (1/1, 150 mL), F3) dichloromethane (150 mL), F4) dichloromethane /ethyl acetate (1/1, 250 mL), F5) ethyl acetate/methanol (9/1, 300 mL) and F6) methanol (200 mL). Each of these 6 fractions was evaluated in bioassays and also aliquoted for further chemical analyses.

5.3.3 Bioassays

Feeding bioassays were carried out with *S. exigua* caterpillars. For every treatment, 16 young second instar caterpillars were used. Each of them was placed in an individual cell with artificial diet designed for lepidopteran rearing (1 cm^3) supplemented with either dried residues of primary solvent extractions (step 1), or those from the 6 fractions related to the aqueous extract (step 2). To assess the effects of diets on the generalist herbivore, the caterpillars were weighed at the beginning of the assays (day 0) and then every three days, until pupation. The diet was renewed every three days and provided *ad libitum*. The days of both the pupation and the emergence of the adults were also recorded. During the step 1, the diets contained dry residues from the three different solvent extracts (methanol, dichloromethane, water + formic acid 0.1%). These extracts were mixed with an artificial diet (Frontiers) to get a 1:1 fresh plant concentration in the diet. The diet was cooked, maintained at 80°C in a hot water bath, and the dry residues added before solidification of the agar. The following treatments were tested in the step 1 of the experiment: extract with methanol; extract with dichloromethane; extract with water + formic acid 0.1%; made for the two tested plants, as well as pure diet used as control (no residues added). This leads to 7 treatments tested in parallel: *Lycopodium annotinum* - H_2O + formic acid 0.1%; *Lycopodium annotinum* - methanol; *Lycopodium annotinum* - dichloromethane; *Dryopteris filix-mas* - H_2O + formic acid 0.1%; *Dryopteris filix-mas* - methanol; *Dryopteris filix-mas* - dichloromethane; and control diet. The same experimental setup was used during the step 2, except that the extracts were replaced by 6 different fractions obtained from the harmful aqueous extract of *Dryopteris filix-mas*, and compared to a non-supplemented control diet.

5.3.4 Chemical analyses of the plant extracts and fractions

Nuclear magnetic resonance spectroscopy (NMR) was used to identify both the chemical components of the *L. annotinum* clubmoss and *D. filix-mas* fern extracts and those containing within the six fractions from the fern aqueous extract. A Bruker Avance Neo Ascend 600MHz analytical device was used with the Mnova NMR software package for data processing (v.14.2.0, MestReLab Research, Santiago de Compostela, Spain).

5.3.5 Statistics

Mass at day 12 of development, pupa mass, duration of development until pupation (days), time from pupation to adult emergence (days) were used as caterpillar performances' parameters in feeding bioassays. ANOVA statistical tests were carried out on these data, followed by Tukey's tests for multiple comparison in order to find means that significantly differ from each other. If the application conditions were not met, Kruskal-Wallis tests followed by post-hoc tests were performed.

5.4 Results

In the step 1 of the experiment, solvent extractions carried out on the *L. annotinum* clubmoss and *D. filix-mas* fern result in overall amounts ranging from 4.4 to 30.8 % of the dry plant mass (Table 1). In the second step, for the fern only, extracts collected in the six different fractions showed from 0.035 to 0.164 % of the total mass of the dry tissues (Table 2). Detailed total masses of plants extracted and subsequent extracts if applicable are shown in the supplementary table 1 (see Supplementary material). In the step 1 of the feeding bioassays, caterpillar masses at day 12 of the development were significantly lower when fed on diets containing either water + formic acid 0.1% (average 2.58 ± 0.88 mg) or methanol (average 16.55 ± 8.59 mg) extracts from the *D. filix-mas* fern. All other treatments, including all the *L. annotinum* clubmoss extracts, do not differ from larval mass at day 12 observed in the control pure artificial diet (average 143.35 ± 22.61 mg) (ANOVA; F= 29.454; p-value < 0.001; Fig. 1). The mortality rate at day 12 followed the same pattern, with caterpillars fed on each of these two diets showing 75% of death during the experiment, against an average 28.3% on the five other diets (Fig. 1). Again, these two fern extracts added to the artificial diets were responsible for annihilating both pupation and adult emergence (0% in fern water + formic acid 0.1%; 12.5% pupation in fern methanol but without any adult emergence), against an average 37.5% (pupation rate) and 25% (adult emergence) seen when fed with pure diet, and the highest 56.25% (pupation rate) and 50% (adult emergence rate) in dichloromethane clubmoss extract (Fig. 2). No significant difference was found regarding the mass of the pupae, neither the survival rate at the pupation stage (ANOVA; F= 2.526; p-value = 0.051; Fig. 3). Mostly diets containing fern extracts increased the average development time until pupation (for instance, 23 days when fed with fern methanol against 15 on pure diet), however important caterpillar mortality in these treatments limited the corresponding statistical tests (Kruskal-Wallis; Chi^2= 24.848; p-value < 0.001; Fig. 4). Herbivores fed with the diet supplemented with clubmoss-dichloromethane extract were the fastest to achieve pupation and to emerge as adults, directly linked to the rapid development time until pupation already observed (Kruskal-Wallis; Chi^2= 20.742; p-value < 0.001; Fig. 5 and Fig. 4).

Regarding the results obtained in the step 1 of the experiment, the most detrimental extract against *S. exigua* herbivores was selected to be part of the step 2. The *D. filix-mas* fern global aqueous extract was then split in 6 fractions, then added each to pure artificial diet before to be tested in another feeding assays using herbivorous caterpillars. As a seventh treatment, pure diet (i.e. without any added plant material), was used as a control.

Residues from the different fractions represented 0.04 to 0.16% of the dry plant mass (Table 2). The detailed total masses of extracted fractions are available in the supplementary table 2 (see Supplementary material). The expected negative effects of the 6 tested fractions were monitored using most of the same indicators as those in step 1. Compared to the pure diet (average 84.02 ± 15.79 mg) significant lower larval masses were observed at day 12 in caterpillars fed with fraction 3 (average 36.04 ± 12.19 mg; ANOVA results: $F_{(6;91)}$=3.864; p-value = 0.002; Fig. 6). Fractions 2, 4, and 6 seem to limit mass gain as well, but remaining statistically not significant. As in step 1, mortality was monitored at day 12 (Fig. 6), nevertheless this measurement showed to be less relevant as more than 80% of all the caterpillars were still alive, no matter the diet. So, the survival rate on the entire 33 days of the experiment was considered (Fig. 7). With 50% of the *S. exigua* herbivores alive at day 33, the fraction 3 was responsible for the poorer survival rate among all the treatments, followed by fraction 4 (58%), whereas fraction 1 allowed the highest 96% survival (Fig. 7).

No difference was observed neither about the pupal mass of *S. exigua* after feeding on diet supplemented with different fractions of aqueous + 0.1% formic acid extract from *D. filix-mas* (ANOVA results: $F_{(6;72)}$= 1.928; p-value = 0.089; Fig. 8), nor on the duration of development time to reach this stage (ANOVA results: $F_{(6;72)}$= 1.939; p-value = 0.086; Fig. 9). However, the caterpillars fed with the fraction 3 showed the lowest percentage of pupation among all the treatments (50%, Fig. 9). Interestingly, fractions 3, 4, and 6 used in diets significantly extended the time need (average 29.42 ± 0.73 days, against a mean 27.22 ± 0.56 days in other diets) to finalize pupation and emerge as an adult (ANOVA results: $F_{(6;72)}$= 2.478; p-value = 0.032) (Fig. 10). With both 50% in adult emergences, fraction 3 and 4 appeared to be the most detrimental diet compared to all the other treatments, whereas fraction 1 and 5 showed the best success (both 75%) (Fig. 10).

None of the 6 fractions was able by itself to trigger equivalent harmful consequences to generalist caterpillar development and survival when compared to the step 1 aqueous + 0.1% formic acid extract of *Dryopteris filix-mas*, and at least 50% of the *S. exigua* herbivores were still alive after 33 days of the experiment (Fig. 7). As the fraction 3 (solvent extraction with dichloromethane alone) showed the strongest negative effects on mass gain (Fig. 6), survival rate (Fig. 7), delayed pupation time or adult emergence (Fig. 10), its main active compounds were tentatively identified analyzed using nuclear magnetic resonance spectroscopy (NMR). The table 3 shows all the phloroglucinols detected in this *D. filix-mas* fern extract, including C_{22}, C_{23}, and C_{24} compounds like flavaspidic acids, desaspidins, margaspidins, aspidins and albaspidins (Table 3).

5.5 Tables and figures

Table 1: Percentage of the total mass of dried plant tissue extracted using three solvent types (methanol, dichloromethane, and water + formic acid 0.1%) in *Lycopodium annotinum* clubmoss and *Dryopteris filix-mas* fern.

Plant	**Solvent**	**% of extract in the dried plant**
Lycopodium annotinum	Dichloromethane	5.70
	Water + formic acid 0.1%	23.54
	Methanol	13.37
Dryopteris filix-mas	Dichloromethane	4.40
	Water + formic acid 0.1%	25.98
	Methanol	30.77

Table 2: Respective percentages of the total mass of dried plant tissue found in six different fractions obtained from an aqueous (water + formic acid 0.1%) *Dryopteris filix-mas* extraction.

Fraction	**Solvent(s) (ratio v/v)**	**% of fraction in the dried plant**
F 1	Heptane	0.064
F 2	Heptane / dichloromethane (1/1)	0.035
F 3	Dichloromethane	0.060
F 4	Dichloromethane / ethyl acetate (1/1)	0.036
F 5	Ethyl acetate / methanol (9/1)	0.164
F 6	Methanol	0.120

Table 3: Tentative identifications and chemical formulas of the active phloroglucinol compounds present in the damaging fraction 3 of the aqueous + 0.1 % formic acid extract of *Dryopteris filix-mas*. In the chemical analyses, C_{22} compounds were twice more abundant than C_{23} that were in turn twice more abundant than C_{24} ones.

Name	**Rough formula**
Flavaspidic acid AB	$C_{22}H_{26}O_8$
Desaspidin AB	$C_{22}H_{26}O_8$
Margaspidin AB	$C_{22}H_{26}O_8$
Norflavispidic acid AB	$C_{22}H_{26}O_8$
para-Aspidin AB	$C_{23}H_{28}O_8$
Albaspidin PP	$C_{23}H_{28}O_8$
Flavaspidic acid BB	$C_{24}H_{30}O_8$
Flavaspidic acid	$C_{24}H_{30}O_8$
Desaspidin BB	$C_{24}H_{30}O_8$
Albaspidin BP	$C_{24}H_{30}O_8$
Margaspidin BB	$C_{24}H_{30}O_8$
Norflavispidic acid BB	$C_{24}H_{30}O_8$

Figure 1

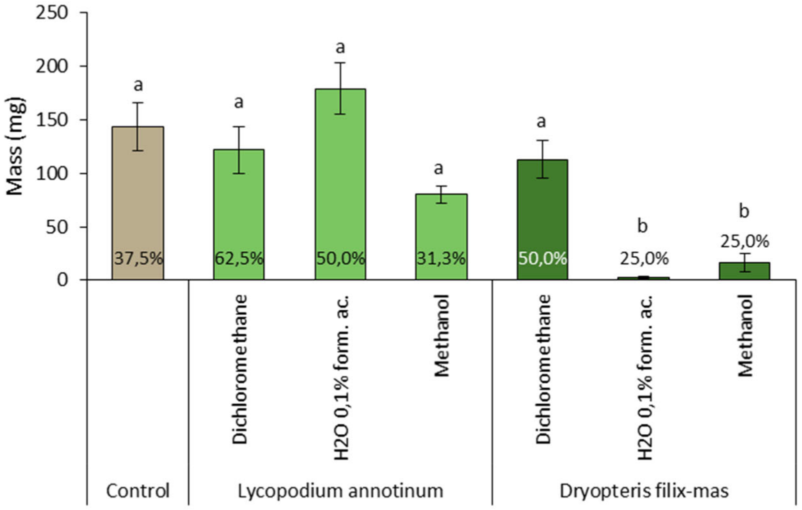


Figure 1: Mass at day 12 (mean ± SE) of *Spodoptera exigua* caterpillars fed with artificial diet supplemented with different extracts of *Lycopodium annotinum* clubmoss (light green), *Dryopteris filix-mas* fern (dark green), or pure diet (control, in brown). Above lettering indicates significant differences according to Tukey test after ANOVA (F= 29.454; p-value < 0.001). Caterpillar survival rate (in %) at day 12 for each treatment are shown close to the y axis.

Figure 2

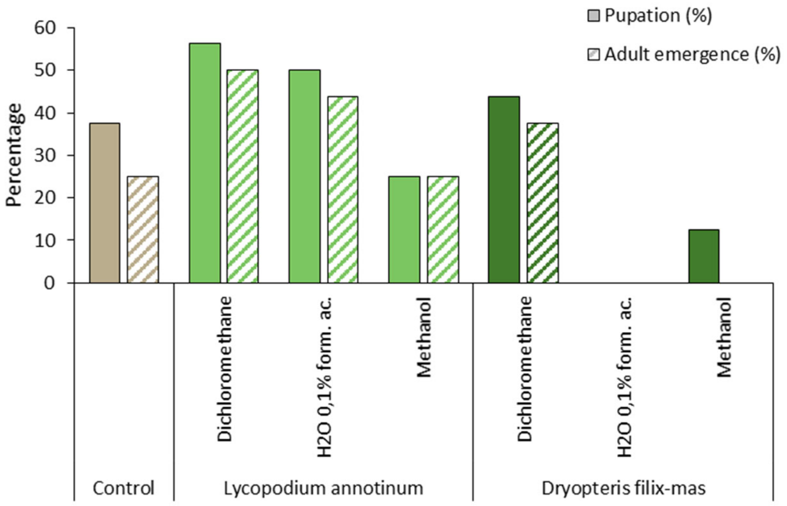


Figure 2: Pupation (plain bars) and adult emergence (dashed bars) rates of *Spodoptera exigua* caterpillars fed with diets supplemented with different extracts of *Lycopodium annotinum* clubmoss (light green), *Dryopteris filix-mas* fern (dark green), or pure used as control (brown).

Figure 3

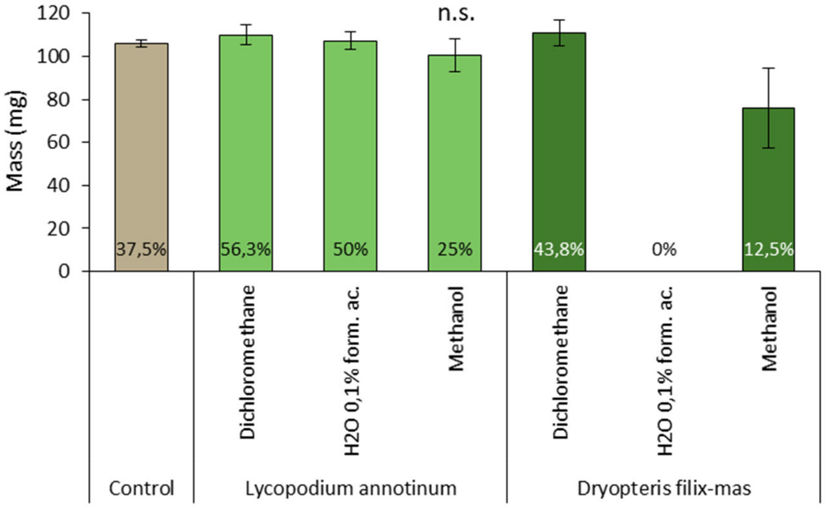


Figure 3: Comparison in masses of *Spodoptera exigua* pupae fed with artificial diets supplemented with extracts of *Lycopodium annotinum* clubmoss (light green), *Dryopteris filix-mas* fern (dark green), or non-supplemented diet used as control (brown) (ANOVA; F= 2.526; p-value = 0.051). Pupae survival rate (in %) for each treatment are shown close to the y axis.

Figure 4

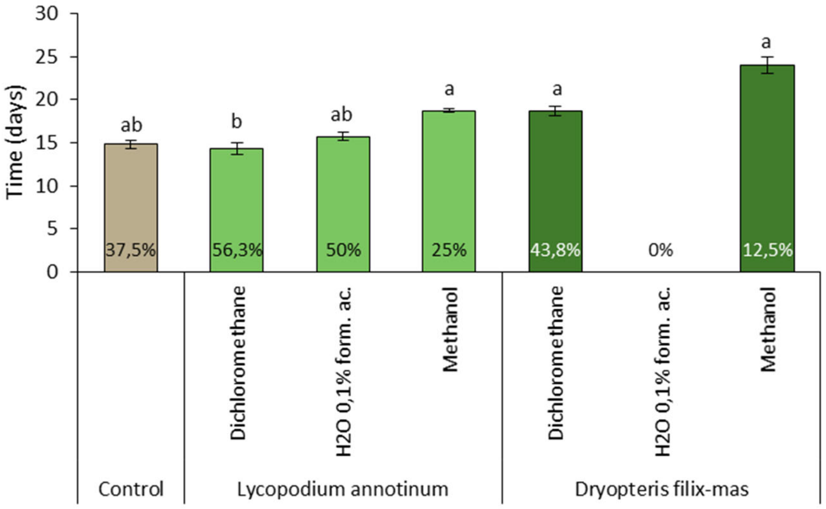


Figure 4: Mean duration of development until pupation (in days ± SE) of *Spodoptera exigua* caterpillars fed with artificial diet supplemented with extracts of *Lycopodium annotinum* clubmoss (light green), *Dryopteris filix-mas* fern (dark green), or non-supplemented diet used as control (brown)(Kruskal-Wallis; Chi^2= 24.848; p-value < 0.001). Letters above indicate significant differences according to post-hoc test. Overall herbivore survival rate (in %) for each treatment are shown close to the y axis.

Figure 5

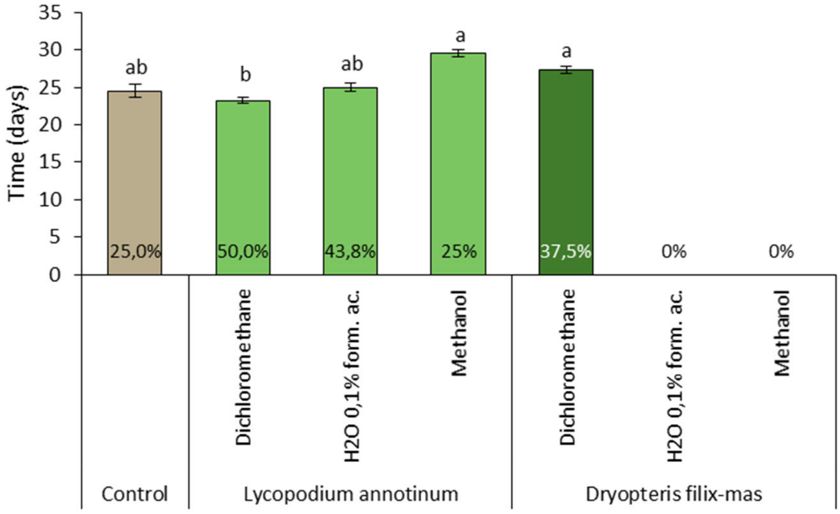


Figure 5: Average time required from pupation to adult emergence (in days ± SE) of *Spodoptera exigua* caterpillars reared on artificial diet supplemented with extracts of *Lycopodium annotinum* clubmoss (light green), *Dryopteris filix-mas* fern (dark green), or non-supplemented diet used as control (brown). Letters above indicate significant differences according to post-hoc test after Kruskal-Wallis (Chi^2= 20.742; p-value < 0.001). Successful adult emergence rate (in %) after pupation and for each treatment are shown close to the y axis.

Figure 6

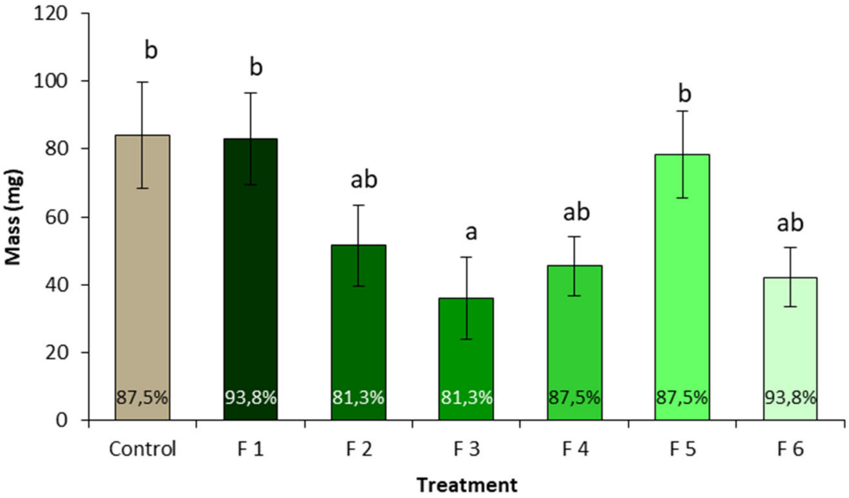


Figure 6: Larval mass of *Spodoptera exigua* caterpillars (mean ±SE) after 12 days feeding on diet supplemented with six different fractions (F1 to F6) of aqueous + 0.1% formic acid extract from the fern *Dryopteris filix-mas*). Letters above the bars are Tukey post-ANOVA test results (ANOVA; $F_{(6;91)}$=3.864; p-value = 0.002). Caterpillar survival rate (in %) at day 12 for each treatment are shown close to the y axis.

Figure 7

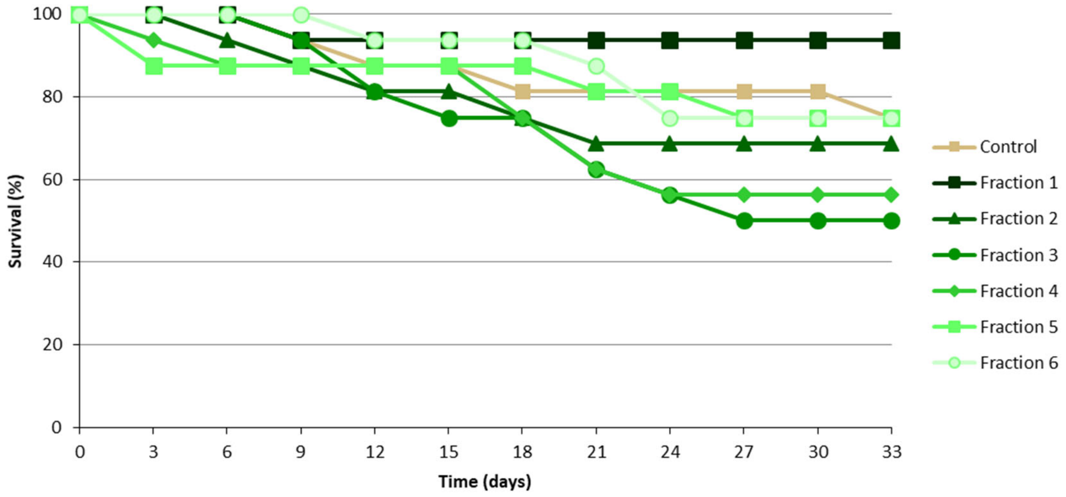


Figure 7: Survival rate (in %) of *Spodoptera exigua* caterpillars over 33 days when fed with 7 different diets, 6 artificial diets mixed with one of the fractions of an aqueous + 0.1% formic acid extract of *Dryopteris filix-mas*, and one without any added plant material used as control (in brown). For each treatment n= 16 caterpillars.

Figure 8

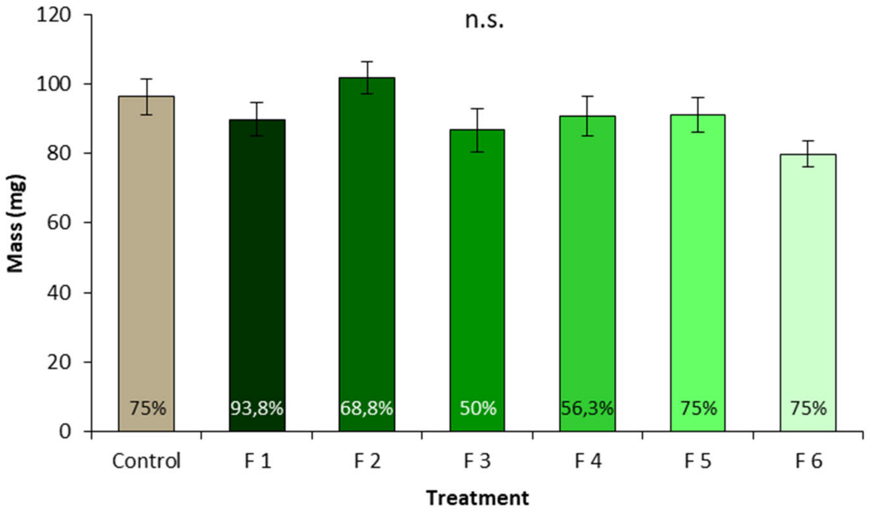


Figure 8: Mass of *Spodoptera exigua* pupae (mean ±SE) after feeding on diet supplemented with six different fractions of aqueous + 0.1% formic acid extract from the fern *Dryopteris filix-mas* (F1 to F6), and one pure diet (brown). ANOVA was performed on log-transformed data (ANOVA results: $F_{(6;72)}$= 1.928; p-value = 0.089). Pupation rates (in %) for each treatment are shown close to the y axis.

Figure 9

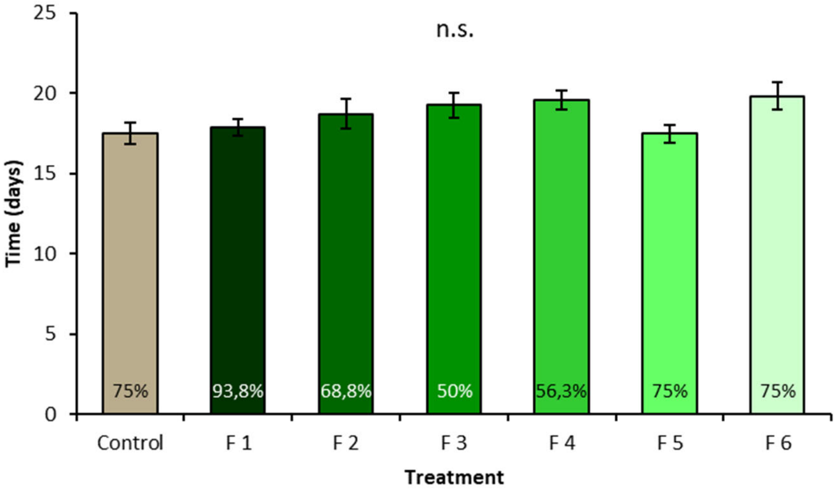


Figure 9: Mean duration of development until pupation (days ± SE) of *Spodoptera exigua* caterpillars after feeding on diet supplemented with six different fractions of aqueous + 0.1% formic acid extract from the fern *Dryopteris filix-mas* (F1 to F6), and one pure diet (brown). ANOVA was performed on log-transformed data (ANOVA results: $F_{(6;72)}$= 1.939; p-value = 0.086). Pupation rates (in %) for each treatment are shown close to the y axis.

Figure 10

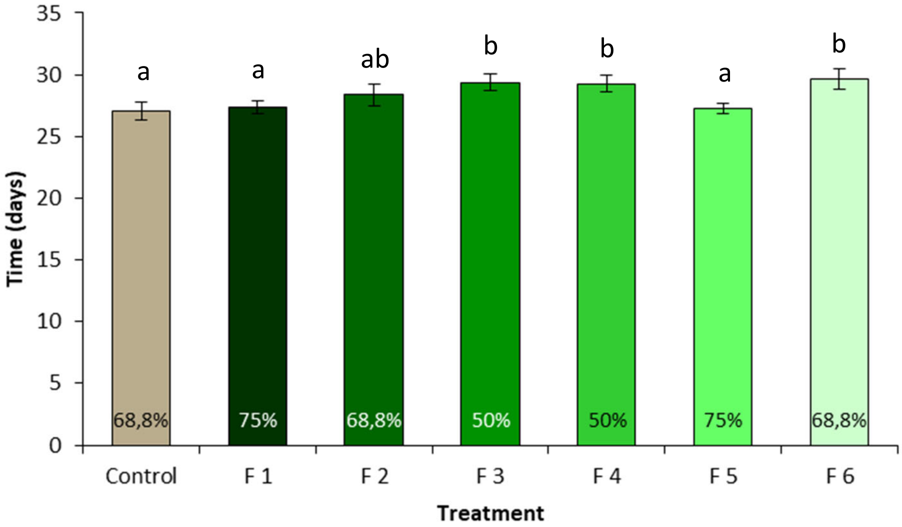


Figure 10: Mean time from pupation to adult emergence (days ± SE) of *Spodoptera exigua* insects after feeding on diet supplemented with different fractions of aqueous + 0.1% formic acid extract from the fern *Dryopteris filix-mas* (F1 to F6), and one pure diet (brown). ANOVA was performed on log-transformed data (ANOVA results: $F_{(6;72)}$= 2.478; p-value = 0.032). Adult emergence rates (in %) for each treatment are shown close to the y axis.

5.6 Discussion

5.6.1 Developmental performances and survival of the generalist herbivore *S. exigua*

Regarding the mass gain, survival rate, developmental time and adult emergence, we observed that *S. exigua* caterpillars developed and survived just as well on the diets containing *Lycopodium annotinum* clubmoss extracts, as on pure artificial diet (Fig, 1, 2, and 3). In contrast, the water + formic acid 0.1% and the methanol extracts from the *Dryopteris filix-mas* fern negatively affected the caterpillars' performances, which was not the case for the dichloromethane extract (Fig. 1, 2, 3, and 5). These results imply that constitutive chemical defenses in some, but not all, early vascular plant species effectively contribute to the plants' resistance to generalist insect herbivores. *L. annotinum* is known to contain certain alkaloids (Orechoff, 1934, Anet and Eves, 1958, Harrison et al., 1961, Koyama et al., 2005, Ishiuchi et al., 2009), but none of them appear to affect the herbivore in our study (Fig. 2). As *Lycopodium* alkaloids usually show only moderate detrimental effect on herbivores, (Olafsdottir et al., 2013), we may assume that the very polyphagous *S. exigua* can tolerate this weak defense. Beside alkaloids, other secondary metabolites are expected to be part of clubmoss chemical defenses. Here, it is however crucial to point out that during the process of making the different diets, an 80°C temperature is reached for a short moment. It is theoretically possible that during this brief temperature certain molecules break down, but Dymek et al. (2021) extracted and studied *Lycopodium* alkaloids under the same 80°C temperature burst without noticing any fundamental change. As we found an effect of the extracts on caterpillar performance, we can assume that if not all, at least many of the active defensive compounds remain functional when added to the artificial diet.

The methanolic and the aqueous extracts from the *Dryopteris filix-mas* leaves negatively impacted the developmental performances and survival of *S. exigua* caterpillars. These extracts were responsible for lower caterpillar masses at day 12 (Fig. 1) and no adult finally emerged after both treatments (Fig. 5). As their polarity is similar, there is certainly an overlap in the compounds extracted by these two solvents (Anyasor et al., 2010), so the similar effects on herbivore development were quite expected. Water-based extraction usually reveals certain aspects about phytochemicals, but methanol is commonly preferred for extraction for subsequent analytical purposes (Vuong et al., 2013, Wang et al., 2019).

The next step of the study focused on the most active fern extract, namely water + formic acid 0.1%. Using a silica column and different solvent combinations (Table 2) six fractions were obtained and tested individually in caterpillar feeding assays. Fraction 3 (dichloromethane), and to some extent 4 (dichloromethane / ethyl acetate), mixed in artificial diets significantly reduced the herbivore's performance, especially in terms of both mass gain at day 12 and survival rate through the 33 days of the experiment (Fig. 6, 7, and 10). However, none of the six fractions triggered the equivalent harmful effect on caterpillar development and survival as compared to the step 1 aqueous + 0.1% formic acid extract of *D. filix-mas*. No matter what diet was offered, at least 50% of the *S. exigua* individuals (caterpillars, pupae or adults) were still alive after 33 days (Fig. 7). Lack of plant material prevented us from recombining all the six fractions, to establish if together they would provoke the stronger effect seen in step 1 of the experiment. Because fraction 3 (solvent extraction with dichloromethane alone) showed the strongest negative effects on mass gain (Fig. 6), survival rate (Fig. 7), and delayed pupation time or adult emergence (Fig. 10), it was selected for further chemical analyses.

Overall, the feeding assays confirm the role and the importance of constitutive chemical defenses in some species of pteridophytes. This suggests that a chemical defenses hypothesis, more than an unlikely “apparent-biomass” (Noy-Meir, 1975) or a recently refuted “poor nutrients” hypothesis (Duhin et al., 2022) is mainly responsible for the virtual absence of herbivores on pteridophytes.

5.6.2 Chemical analyses of the plant’s extracts and fractions

To isolate and identify possible defense compounds, the aqueous extract of *D. filix-mas* fern was split in six fractions, then individually tested in mixed diets used for the caterpillar feeding assays (Table 2). As the fraction 3 (dichloromethane) showed the most potent activity against *S. exigua*, its main compounds were identified using NMR, revealing the presence of phloroglucinols compounds (Table 3). Among them, flavaspidic acids as filicin (or filixic acid) was previously isolated from *D. filix-mas* in 1845 (Luck, 1845) and was shown to have anthelmintic (antiparasitic) activity (Heikinheimo, 1963). Similarly, desaspidin is known as an anthelmintic that naturally occurs in some ferns, while margaspidin has been identified from *Dryopteris spp.* ferns (Penttilä and Kapadia, 1965). Aspidin is a natural active product found in *Dryopteris fragrans* and albaspidin in *D. villarii* and *D. aitoniana* according to the PubChem open chemistry database of the National Institutes of Health (NIH).
More than 700 different phloroglucinols have been described in a wide range of organisms including plants and microorganisms (Singh and Bharate, 2006). These compounds are commonly found in ferns (Penttilå and Sundman, 1970, Widén et al., 1996, Widèn et al., 1983, Murakami and Tanaka, 1988, Socolsky et al., 2012). They are mainly located in the hairs present on the rhizomes, at the basis of the leaves (Franchi and Ferri, 1988), or in external leaf glands in a few *Dryopteris* species, along with defensive flavonoid aglycones (Wollenweber et al., 1998). The phloroglucinols identified here were previously found in the rhizomes of *D. filix-mas* in Switzerland, in a study that revealed that phloroglucinol levels may vary between fern populations across the world (Euw et al., 1980). The phloroglucinols are recognized defensive compounds that have been intensively studied in herbivory-seed plant interactions, especially involving plants in the *Eucalyptus* genus (Myrtaceae) (Andrew et al., 2007, Freeman et al., 2008). Some ferns have also been studied in this context, showing toxicity against a nematodes and insects (Shukla and Tiwari, 2011, Ali et al., 2012, Benoufella-Kitous et al., 2014, Han et al., 2015, Egorova et al., 2021).

As the overall negative effect of the total aqueous fern extract (step 1) on herbivore performance was not nearly as strong by any of the six fractions used in the second feeding bioassays, we suggest that: 1) the fractioning process was unable to extract all the active compounds in one of the fractions (supplementary table 2), or 2) a single secondary metabolite or one compound family is unlikely to be solely responsible for the suppression of herbivore performance. In angiosperms, many synergetic combinations of several compounds have frequently been shown to produce the general negative impact on an herbivore (Nelson and Kursar, 1999, Richards et al., 2016). If sufficient plant material were to be available, it would be interesting to recombine our six fractions to test if it would fully restore the harmful effect on the caterpillars. At least, our findings highlight the important role of phloroglucinols as constitutive defense chemicals of *D. filix-mas*, confirming an early evolutionary emergence of chemical defensive mechanisms in certain vascular plants.

5.6.3 Evolutionary considerations

Phloroglucinols are biosynthesized by numerous organisms, including plant-associated *Pseudomonas fluorescens* bacteria (Achkar et al., 2005, Yang and Cao, 2012, Biessy and Filion, 2021). As phloroglucinols in ferns are mainly found in rhizomes, it is hypothesized that plants may have horizontally acquired specific genes involved in phloroglucinols production from the bacteria through their usual interactions, as has been the case for higher plants (Intrieri and Buiatti, 2001). Molecular approaches are now focusing on genetic transformation efforts to produce phloroglucinols in model plants and crops, aiming at enhanced protection against pathogens and pest herbivores (Fenton et al., 1992, Dowling and O'Gara, 1994, Abdel-Ghany et al., 2016).

In a broader evolutionary context, early vascular plants have coexisted since a very long time with herbivorous insects and other herbivores, as evident from some fossil records (Labandeira, 2007, Robledo et al., 2015). Our finding that that the negative effect of *D. filix-mas* on a general herbivory relies on toxic chemicals produced in fronds, reveals that early vascular plants must have developed primary defensive mechanisms millions of years ago, well before the gymno- and angiosperm supremacy seen nowadays (Wittstock and Gershenzon, 2002).

5.7 Conclusion

Using different pteridophytic species, the generalist caterpillar *S. exigua*, feeding bioassays and analytical chemistry, this study shows that early vascular plants and in particular the fern *D. filix-mas* may use chemical defenses to significantly reduce herbivory. Millions of years ago, ancestors of these plants must have developed defensive mechanisms against both abiotic and biotic stresses. These adaptations must have contributed to the wide array of defenses observed in the modern plants. The mechanisms behind the chemical defenses in early vascular plants are poorly understood, and deserve further attention. This seems particularly important in the context of global change context, if we wish to understand how plant species might adapt or perish.

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5.10 Supplementary material

Supplementary table 1: Masses (in g) of the extracts of *Lycopodium annotinum* and *Dryopteris filix-mas* obtained with three different solvents (methanol; dichloromethane; and water + formic acid 0.1%) from dried plant tissue.

Plant	Solvent	Mass of plant used (g)	Mass of extract (g)
Lycopodium annotinum	Dichloromethane	60.8554	3.472
	Water + formic acid 0.1%	61.5969	14.500
	Methanol	59.6935	7.984
Dryopteris filix-mas	Dichloromethane	24.2875	1.068
	Water + formic acid 0.1%	44.8124	11.641
	Methanol	39.0664	12.019

Supplementary table 2: Masses (in mg) of the fractions made from the aqueous (water + formic acid 0.1%) extract of *Dryopteris filix-mas* (initial mass of plant used = 44.81 g, initial mass extracted = 11.64 g (see supplementary table 1), total mass of the 6 fraction = 214.6 mg).

Fraction	Solvent(s) (ratio v/v)	Mass of fraction (mg)
F 1	Heptane	28.7
F 2	Heptane / dichloromethane (1/1)	15.7
F 3	Dichloromethane	27.0
F 4	Dichloromethane / ethyl acetate (1/1)	16.0
F 5	Ethyl acetate / methanol (9/1)	73.3
F 6	Methanol	53.9

6 Chapter 4: Volatile emissions and hormonal changes of the fern *Polypodium vulgare* in responses to infestation by the specialized aphid *Idiopterus nephrelepidis* and the generalist caterpillar *Spodoptera exigua*

Audrey DUHIN[1], Gaetano TINELLI[1], Gaëtan GLAUSER[2], Ted C. J. TURLINGS[1] and Gregory RÖDER[1]

[1] Laboratory of Fundamental and Applied Research in Chemical Ecology, Institute of Biology, University of Neuchâtel, Switzerland

[2] Neuchâtel Platform in Analytical Chemistry (NPAC), Faculty of Sciences, University of Neuchâtel, Switzerland

6.1 Abstract

Plants under herbivore attack are known to change their emissions of volatile organic compounds (VOCs) through the mobilization of hormonal pathways. This phenomenon is poorly studied in early land plants like ferns. In this study, we first investigated the interaction between the common polypody fern (*Polypodium vulgare*, Moniliophyta: Polypodiaceae) and a specialized sap-sucking insect, the black fern-aphid (*Idiopterus nephrelepidis* Davis 1909, Hemiptera: Aphididae). No differences in VOCs releases and content of five known plant defense hormones were measured between ferns under aphid attack and undamaged control plants, suggesting that the specialized insect does not induce these kinds of defenses in the fern. By contrast, the beet armyworm (*Spodoptera exigua* Hübner 1808, Lepidoptera: Noctuidae), a generalist leaf-chewing insect, was found to trigger a VOCs emissions pattern that significantly differed from the one of intact control ferns. As a third treatment, mechanically damaged ferns showed only minor changes in volatile emissions compared to control ferns. Interestingly, in all these experiments we found no difference at the phytohormone level among the different treatments. Overall, the VOCs emissions in the common polypody fern suggest that early vascular plants may have evolved the ability to respond to herbivory with the release of volatiles well before the gymno- and angiosperm seed plants. In parallel, fern hormonal or metabolic pathways involved in these responses require more exhaustive investigations aiming to understand of how such early defensive traits evolved throughout the long evolutionary history of plants.

6.2 Introduction

Interactions between herbivores and plants seem unbalanced, with an active and aggressive part played by the former, while the latter looks like as a static prey. In fact, apart from some capacity to passively tolerate herbivory, plants have developed strategies to actively overcome such attacks and defend themselves against various types of herbivores. Active defenses rely on the production of direct physical barriers, such as spines and trichomes (Hanley et al., 2007), and constitutive chemical arsenal overcoming herbivores. As well, defenses can be indirect, inviting a third part to the interaction. In both cases, defenses can be inducible when consecutively produced under attack. For instance, plants are well known to produce volatile organic compounds (VOCs), which can be constitutive and directly released upon damages or can also be induced and emitted in response to a vast range of both abiotic and biotic stresses (Holopainen and Gershenzon, 2010).

The induction of plant defenses is mediated through phytohormonal signaling pathways, including the jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) pathways (Ozawa et al., 2000, Von Dahl and Baldwin, 2007, Farmer et al., 2003). In higher plants lineages (i.e. spermatophytes), chewing insects are mainly responsible for inducing the JA pathway, whereas the sap-sucking insects act mainly on the SA one (Soler et al., 2012, Schweiger et al., 2014). Moreover, cross-talk between signaling pathways may contribute to the final plant response (Walling, 2000, Bostock et al., 2001, Thaler et al., 2012).

In early vascular plants such as pteridophytes (i.e. ferns and allies), these pathways and their interactions have been investigated in the *Pteris* genus, showing possible feedback between JA and abscisic acid (ABA), and an inhibition of JA biosynthesis by SA in one of the three studied species (Zemanová et al., 2019). In parallel, studies showed that the JA pathway can enhance VOCs release in the *Dryopteris filix-mas* fern (Boland et al., 1995). The same result was obtained by Radhika et al. (2012) with the bracken fern *Pteridium aquilinum*. When Radhika et al. (2012) tried to induce VOCs emissions by both a generalist and a specialist herbivore, and by mechanical damage, they could detect only very low quantities of volatiles which were statistically equivalent between the treatments.

Some studies focused on fern volatiles in the context of industrial applications like aroma or perfumery (Fons et al., 2010, Froissard et al., 2014, Froissard et al., 2011), providing a first insight of which volatile compounds are released by some fern species. Role and importance of such volatiles in likely fern-herbivore interactions remain overall unknown, whereas the production of VOCs after a drought abiotic stress has been investigated in the rustyback fern *Asplenium ceterach* (Živković et al., 2021).

In recent studies, VOCs produced in various species of the Polypodiaceae family were identified for the first time and showed typical smell within the investigated genera (Kessler et al., 2015a), whereas key insect-induced VOCs released by the same ferns were identified as (E)-2-hexene and (E)-2-pentenol (Soriano and Clavijo-McCormick, 2020).

The possible link between VOCs emissions and phytohormones in the early vascular plants has partially been investigated. These hormones mediate plant defensive reactions against various stresses, as herbivory. Under attack, different phytohormones take part into a combined signaling pattern allowing appropriate plant responses (Walling, 2000, Bostock et al., 2001). Radhika et al. (2012), using the bracken fern *Pteridium aquilinum* as model, were unable to establish a clear relation between intrinsic phytohormone production by a fern and a change in the subsequent emission of VOCs. Nevertheless, a direct application of JA on mechanically wounded fern fronds leads to significant changes in VOCs emissions (Radhika et al., 2012, Soriano and Clavijo-McCormick, 2020).

Phytohormones have many roles in the plant physiology. Auxins, cytokines, gibberellins, ABA and ET are mainly involved in growth regulation. Auxins and ABA are known to have a role in abiotic stress response as well. Both JA and SA have to be considered as they interact with the phytohormones above-mentioned. Succinctly, JA is involved in the plant response to chewing herbivores and necrotrophic pathogens, while SA as well as ET operate against biotrophic pathogens (Verma et al., 2016). The JA pathway relies on intermediate compounds including 12-oxophytodienoic acid (OPDA) and jasmonate-isoleucin (JA-Ile), a precursor and a subproduct, respectively (Balbi and Devoto, 2008).

In this study we compared both the phytohormonal reactions and the volatile emissions of the common polypody fern (*Polypodium vulgare*) under 4 different treatments, namely 1) herbivorous attack by the specialist sap-sucking aphid *Idiopteris nephrelepidis,* 2) attack by the generalist leaf-chewer *Spodoptera exigua* (beet armyworm), 3) mechanically wounded, and 4) healthy used as control, contributing to fill the gap in scientific knowledge about defensive mechanisms in early land plants.

6.3 Material and methods

6.3.1 Plants

Wild common polypody ferns (Moniliophyta: Polypodiaceae: *Polypodium vulgare* L.) were collected from three different populations around Neuchâtel (Switzerland) in May, June and July 2020 for the experiments with specialized aphids, and in May and June 2021 for both generalist leaf-chewing herbivory and mechanical damages. All plants with developed fronds were individually placed in small cylindrical plastic pots (4 cm in diameter, 11 cm high) fitting with the volatile collection system available in the laboratory (Turlings et al., 1998), and kept under controlled conditions in a growth chamber (CLF Plant Climatics) as follows: 13-15°C; 70% RH; day/night photoperiod of 12:12hrs during at least two weeks before any of the experiments.

6.3.2 Insects

6.3.2.1 Specialized sap-sucking aphid

Idiopteris nephrelepidis aphids Davis 1909 (Hemiptera: Aphididae) were initially collected from a separate wild fern population (*i.e.* not included in the further experiments) (Neuchâtel, Switzerland). All the aphids were kept in the laboratory on a mix of fern species. The colony was allowed to reproduce sexually and non-sexually freely in the rearing. Wingless adult individuals only were used for the experiments.

6.3.2.2 Generalist chewing caterpillar

Spodoptera exigua Hübner 1808 (Lepidoptera: Noctuidae) caterpillars were used as a generalist herbivore. They were obtained from a rearing facility at the University of Neuchâtel where they are maintained on artificial diet designed for lepidoptera rearing (Frontiers).

6.3.3 Treatments to induce plant defense responses

The plants were treated for 24 hours as followed: 1) infested with 10 aphids; 2) infested with one third stage caterpillar that was starved for 24 hours prior the start of the experiment, then placed in a clip cage of 4.52 cm2 (BioQuip Product, USA) on a fern frond; 3) mechanically damaged with scissors by making a total of 3 cuts in distinct leaflets; or 4) healthy undamaged used as a control.

6.3.4 VOCs collection and analysis

The volatile organic compounds (VOCs) released by each fern placed in an individual glass vessel were collected for 24 hours as described by Turlings *et al.* (1998). In the VOCs collection system, an inflow of humidified and pressured air cleaned over a charcoal filter entered (0.6L/min) into the vessel containing the plant, whereas a diaphragm pump (ME2 Vacuubrand, Germany) pulled out (0.4L/min) the headspace above the fern through an adsorbent tube filled with Tenax-TA polymerous material for trapping. Collections were made in two consecutive experiments, a year apart: the first set involved 6 consecutive batches of 8 aphid-infested and 4 control plants, resulting in a total of 48 aphid-infested and 24 control fern samples, the second set included 6 batches as well with 4 plants per treatment leading to 24 ferns attacked by chewing caterpillars (*S. exigua*), 24 mechanically damaged ferns, and 24 undamaged control ferns.

Right after a batch collection, all the adsorbent tubes containing the VOCs were hermetically stored in a tray where a multipurpose robotic sampler (MPS, Gerstel GmBH) transfer them one at a time in gas chromatograph (Agilent 7890a) coupled with a mass spectrometry detector (Agilent 5975c) (GC-MS) for chemical analyses. Each tube was individually desorbed in a thermal desorption unit (TDU Gerstel, GmBH) (50°C-250°C initial-final temperatures, at a ramp of 60°C/min, hold 3.5 min, splitless mode). All the desorbed VOCs were cryofocused at -80°C (CIS Gerstel, GmbH), before to be simultaneously released into an Agilent HP5-MS separative column (30m x 0.25um x 0.25mm) with a ramp of 120°C/min to reach 260°C, hold time 6 min, PTV solvent mode injection (14 psi) Helium was used as the carrier gas at 0.9 mL/min constant flow into the column. GC program ran an initial temperature of 40°C (hold 3.5 min), then a ramp at 6°C/min until 260°C hold for 2 min. A mass scan over the range 35-350 m/z and an electron ionization at 70 eV were set in the MS quadrupole detector. MS source and quad temperatures were set at 230°C and 150°C, respectively. Tentative identifications of the compounds were based on cross search using both NIST21 library and the PBM quick search databases available from the Agilent ChemStation data analysis software. Carried out on the same analytical system and with the same settings, a calibration curve using different quantities (45, 90, 180 ng) of pure linalool (Sigma Aldrich) mixed in one µL of dichloromethane was used to quantify the VOCs released by the ferns. This calibration curve based on a compound related to those mainly found in the fern emissions allows to have a pertinent quantitative tool without interfering with the VOCs trapped in the adsorbent tubes, and then was preferred to an added internal standard before analysis. Final quantities were converted in ng emitted per cm^2 of fern frond (leaf area), based on the scan and area measurements of each polypody fern frond included within this study (Adobe Photoshop, CC version, software).

6.3.5 Phytohormone measurements

The following first five (aphid experiment) or all the six (caterpillars and mechanical damage experiment) phytohormones were measured according to the method described in Glauser et al. (2014): salicylic acid (SA); jasmonic acid (JA); jasmonate-isoleucin (JA-Ile), 12-oxophytodienoic acid

(OPDA); abscisic acid (ABA) and indole-3-acetic acid (IAA). At the end of the 24 hours VOCs collection, all the plants were harvested and frozen in liquid nitrogen. The samples were kept at -80°C before being ground under liquid nitrogen. Around 100 mg of plant material were exactly weighed in tubes and extracted twice with ethyl acetate:formic acid (99.5:0.5 v:v) solution, containing internal radiolabeled hormone standards (10 ng/mL of d_5-JA, d_6-ABA, d_6-SA, d_2-GA3, $^{13}C_6$-JA-Ile and 1 µg/mL of d_5-IAA). The extraction was carried out with the addition of a mixer mill and glass beads. The supernatant was then evaporated in a speed-vac system and the dry extract resuspended in methanol. This solution was transferred in an adapted vial and analyzed with an UHPLC-MS/MS machine (UHPLC system coupled to a Xevo TQ-XS triple quadrupole mass spectrometer (Waters, USA); Acquity BEH C18 column (2.1 × 50 mm i.d., 1.7 µm particle size) at 35°C). The data obtained were converted to ng of hormone per mg of fresh plant materiel.

6.3.6 Statistical analyses

The statistical analyses were performed using the R software (R version 4.1.2). The VOCs emissions as well as the phytohormones contents were processed separately for the two respective experiments (i.e. for the aphid-infested plants on one hand, and the caterpillar-infested and mechanically damaged plants on the other hand) each compared with their respective undamaged control ferns. For the aphids vs. control, the data were analyzed using a t-test comparing the total amounts of VOCs and a Wilcoxon signed rank test regarding differences between the treatments in individual VOCs and phytohormones. For the other series with caterpillar-infested, mechanically damaged and control ferns, an ANOVA was used whenever the pre-required conditions were met, otherwise a Kruskal-Wallis test was used for the VOCs and phytohormone data. Those tests were followed by post-hoc tests (Tukey tests for ANOVAs) when the p-value was lower than 0.05 aiming to determine which treatment was significantly different from the others.

6.4 Results

6.4.1 VOCs - aphid-infested vs. undamaged control ferns

Different classes of VOCs were detected in the volatile blends, including aldehydes, ketones, aliphatic alcohols, and terpenoids with mono- and sesquiterpenes (Supp. table 1). There was no statistical difference between the VOCs released by aphid-infested ferns and those of control ferns, neither in the quantities of individual compounds (Wilcoxon tests, p-value > 0.05; Fig. 1, Supp. table 1), nor in the total amount of VOCs (t-test t = 0.677, p-value = 0.501; Fig. 1, Supp. table 1).

VOCs - caterpillar-infested, mechanically damaged and undamaged control ferns

Different classes of VOCs were found, namely 13 aldehydes, eight ketones, seven aliphatic alcohols, 13 terpenoids with mono- and sesquiterpenes, six alkanes, three fatty acids, and one carboxylic ester (Supp. table 2). Here, significant differences were found between the three treatments in the total VOCs released, with plants attacked by caterpillars showing the highest emissions (ANOVA: $F_{(2,57)}$ = 9,535; p-value < 0,001 and Tukey tests' results: caterpillar-control p-value < 0,001; mechanical damage-control p-value = 0.357; and mechanical damage-caterpillar p-value = 0.015; Fig. 2, Supp. table 2). For the individual volatile compounds, they were more abundant in ferns attacked by the caterpillars, then in mechanically damaged plants. Among the 26 compounds which significantly differ (on a total of 51 compounds considered), we observed aldehydes (hexanal; octanal; nonanal; (E)-2-nonenal; benzeneacetaldehyde; decanal; undecanal; dodecanal), terpenes (β-pinene; 1,3,8-p-menthatriene; D-limonene; 3,7-dimethyl-6-octen-1-ol; pinocarvone; geranyl isovalerate; p-menthane-

1,2,3-triol), ketones (2,2,7,7-tetramethyltricyclo[6.2.1.0(1,6)]undec-4-en-3-one; benzophenone), alcohol (isogeraniol), fatty acids (nonanoic acid; n-decanoic acid; n-hexadecanoic acid), and a carboxylic ester (2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester propanoic acid) (Fig. 2, Supp. table 2).

6.4.2 Phytohormones - aphid-infested vs. undamaged control ferns

Among the five phytohormones monitored, SA showed the highest level with around 50 mg/g fresh plant, followed by ABA (around 35 mg/g fresh plant), whereas plants contained low amounts of JA-Ile (around 0.07 mg mg/g fresh plant) (Fig. 3, see Supp. fig. 1 for individually phytohormone optimized scale). However, no significant difference was observed between the two treatments after the 24 hours experiment (Fig. 3).

6.4.3 Phytohormones - caterpillar-infested, mechanically damaged and undamaged control ferns

As for the infestation with aphids, salicylic acid showed the highest amounts, around 22 mg/fresh plant compared to the other phytohormones. Indole-3-acetic acid, which was analyzed in this part of the study only, shows approximately a 5 mg/g of fresh tissue. No significant difference was found between the treatments after 24 hours (caterpillar-induced plants, mechanically damaged plants and control plants) (Fig. 4). For each phytohormone, a detailed plot with statistical parameters is available in the supplementary material (Supp. fig. 2).

6.5 Figures

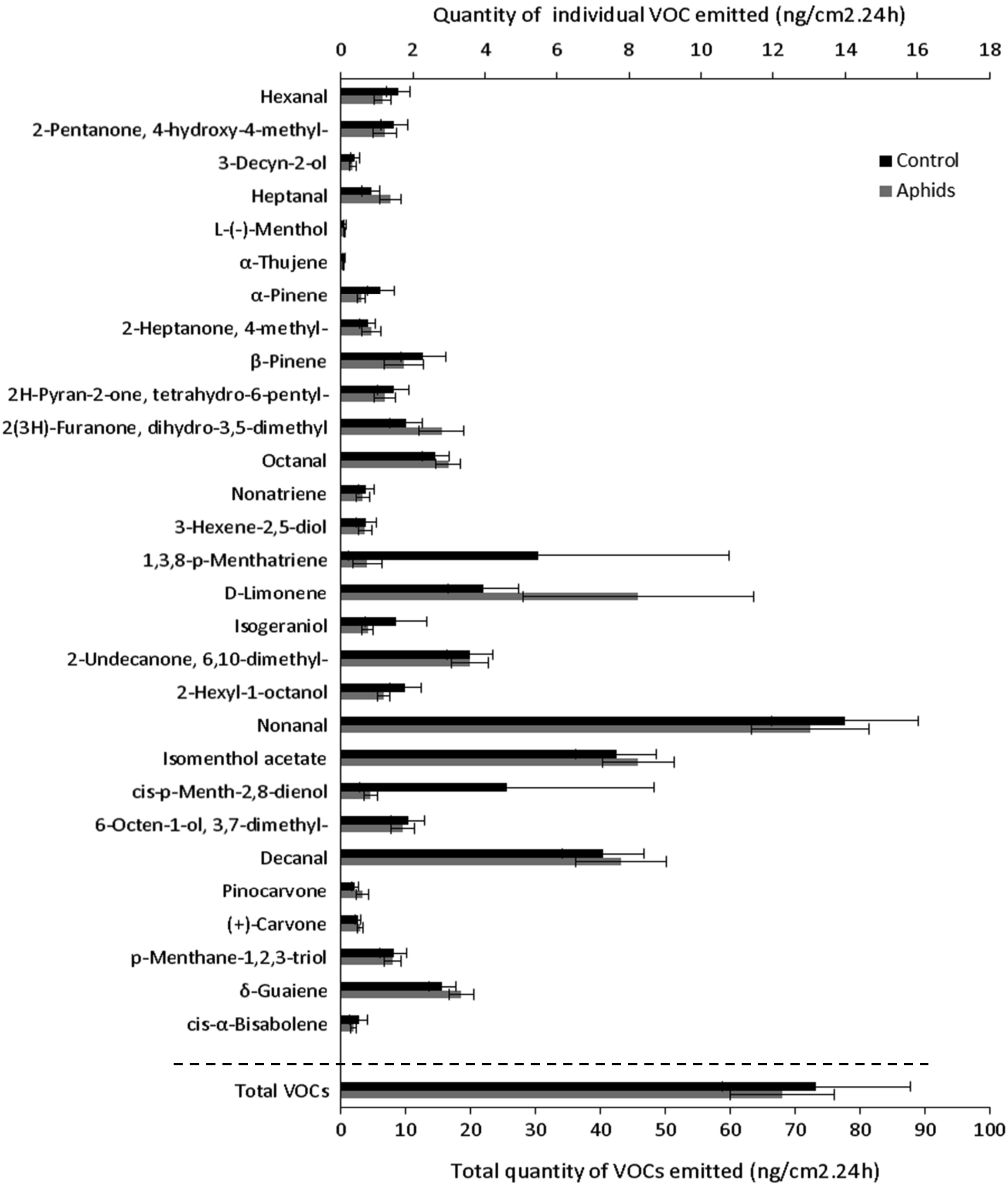

Figure 1: Volatile organic compounds (VOCs) released (ng per cm^2 of frond in 24 hrs; mean ± SE) by *Polypodium vulgare* fern under the attack of sap-sucking *Idiopterus nephrelepidis* aphid (gray bars, n= 48) compared to undamaged control plants (black bars, n= 24). The axis above is for the individual compounds listed on the left and the secondary axis at the bottom is for the total VOCs emitted in both treatments. Using Wilcoxon tests, none of those individual compounds showed any significant statistical difference between the two treatments ($p > 0.05$). No significant difference between the total VOCs was observed either, based on a t-test (log+1 - transformed data, $t = 0.677$, $df = 54.207$, p-value = 0.501).

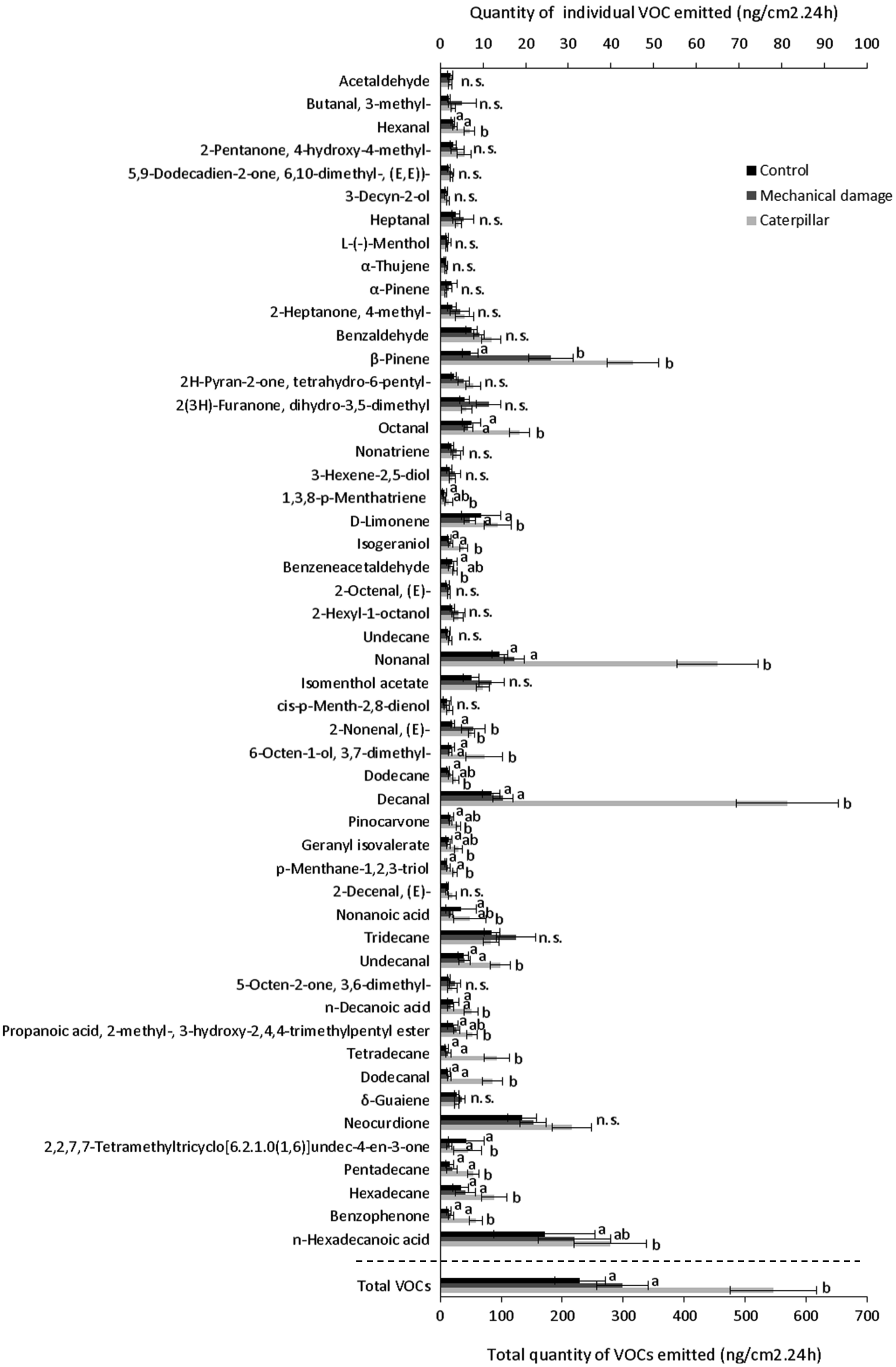
Quantity of individual VOC emitted (ng/cm2.24h)
0
10
20
30
40
50
60
70
80
90
100
Control
Mechanical damage
Caterpillar
Acetaldehyde n.s.
Butanal, 3-methyl- n.s.
Hexanal a a b
2-Pentanone, 4-hydroxy-4-methyl- n.s.
5,9-Dodecadien-2-one, 6,10-dimethyl-, (E,E))- n.s.
3-Decyn-2-ol n.s.
Heptanal n.s.
L-(-)-Menthol n.s.
α-Thujene n.s.
α-Pinene n.s.
2-Heptanone, 4-methyl- n.s.
Benzaldehyde n.s.
β-Pinene a b b
2H-Pyran-2-one, tetrahydro-6-pentyl- n.s.
2(3H)-Furanone, dihydro-3,5-dimethyl n.s.
Octanal a a b
Nonatriene n.s.
3-Hexene-2,5-diol n.s.
1,3,8-p-Menthatriene a ab b
D-Limonene a a b
Isogeraniol a a b
Benzeneacetaldehyde a ab b
2-Octenal, (E)- n.s.
2-Hexyl-1-octanol n.s.
Undecane n.s.
Nonanal a a b
Isomenthol acetate n.s.
cis-p-Menth-2,8-dienol n.s.
2-Nonenal, (E)- a b b
6-Octen-1-ol, 3,7-dimethyl- a a b
Dodecane a ab b
Decanal a a b
Pinocarvone a ab b
Geranyl isovalerate a ab b
p-Menthane-1,2,3-triol a a b
2-Decenal, (E)- n.s.
Nonanoic acid a ab b
Tridecane n.s.
Undecanal a a b
5-Octen-2-one, 3,6-dimethyl- n.s.
n-Decanoic acid a a b
Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester a ab b
Tetradecane a a b
Dodecanal a a b
δ-Guaiene n.s.
Neocurdione n.s.
2,2,7,7-Tetramethyltricyclo[6.2.1.0(1,6)]undec-4-en-3-one a a b
Pentadecane a a b
Hexadecane a a b
Benzophenone a a b
n-Hexadecanoic acid a ab b
Total VOCs a a b
0
100
200
300
400
500
600
700
Total quantity of VOCs emitted (ng/cm2.24h)

Figure 2: Volatile organic compounds (VOCs) released (ng per cm^2 of frond in 24 hrs; mean ± SE) by *Polypodium vulgare* fern under the attack of *Spodoptera exigua* caterpillar (light gray, n= 24), mechanically damaged (middle gray, n= 24) or undamaged used as a control (black, n= 24). The axis above is for the individual compounds listed on the left and the secondary axis at the bottom is for the total VOCs emitted in the three treatments. The differences between treatments of the individual compounds were established with Kruskal-Wallis tests. ANOVA and Tukey tests show the significant differences in the total VOCs emitted between the three treatments (ANOVA: $F_{(2,57)} = 9{,}535$; $p < 0.001$; and Tukey test: caterpillar-control $p < 0{,}001$; mechanical damage-control $p = 0.357$; mechanical damage-caterpillar $p = 0.015$).

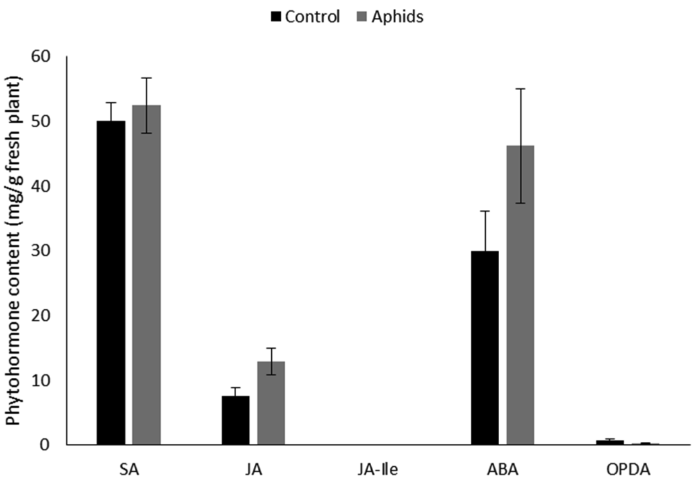

Figure 3: Phytohormone contents (mg/g fresh plant) measured in *Polypodium vulgare* (mean ± SE) after attack by *Idiopterus nephrelepidis* aphids (gray bars, n= 48) and in undamaged control plants (black bars, n= 24). SA = salicylic acid, JA = jasmonic acid, JA-Ile = jasmonate-isoleucin, OPDA = 12-oxophytodienoic acid and ABA = abscisic acid. No statistical difference was observed between the two treatments (Wilcoxon tests, SA (W = 404, p = 0.945); JA (W = 304, p = 0.142); JA-Ile (W = 417, p = 0.774; ABA (W = 344, p = 0.399); and OPDA (W = 440; p-value = 0.364)).

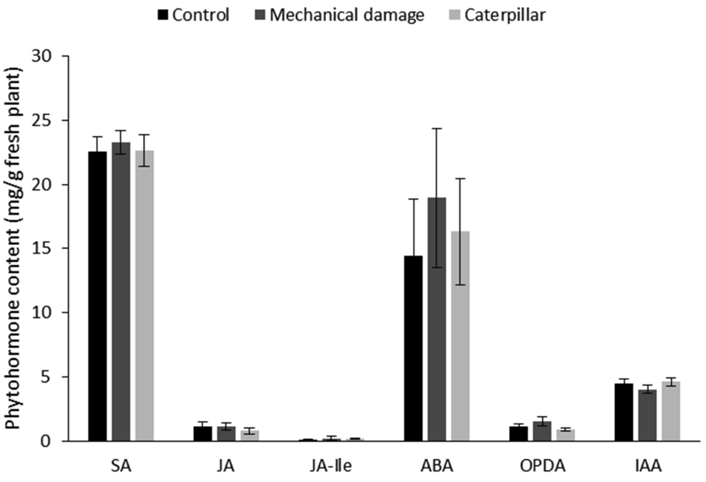

Figure 4: Phytohormones contents (mg/g fresh plant) measured (mean ± SE) in *Polypodium vulgare* after attack by *Spodoptera exigua* caterpillar (light gray, n= 24), mechanically damaged (dark gray, n= 24), and undamaged ferns (black, n= 24). SA = salicylic acid, JA = jasmonic acid, JA-Ile = jasmonate-isoleucin, OPDA = 12-oxophytodienoic acid, ABA = abscisic acid and IAA = indole-3-acetic acid. No statistical difference was observed between the three treatments (ANOVA: SA: $F_{(2;57)}$ = 0.125, p-value = 0.883; JA: $F_{(2;57)}$ = 0.476, p-value = 0.624 (^0.1 -transformed data); JA-Ile: $F_{(2;57)}$ = 1.887, p-value = 0.161 (square root-transformed data); ABA: $F_{(2;57)}$ = 0.233 , p-value = 0.793; OPDA: $F_{(2;57)}$ = 1.701, p-value = 0.192 (log-transformed data); IAA: $F_{(2;57)}$ = 0.810 , p-value = 0.450).

6.6 Discussion

VOCs emissions by early vascular plants like ferns in response to herbivory and the role of phytohormones in it are still mostly understudied. Our results suggest that i) common polypody ferns (*P. vulgare*) can adapt their VOCs emissions, depending on the type of herbivory or damage undergone, and that ii) the five, respectively six, phytohormones considered in this study are unlikely to act as key factors in the defensive plant responses. Similar results were obtained by Radhika et al. (2012) with the bracken fern *Pteridium aquilinum*, as when they tried to induce VOCs emissions by both a generalist and a specialist herbivore, as well as by mechanical damage, observing only very low quantities in all the treatments without any statistical difference. Within the common polypody fern *P. vulgare*, we found that an attack by the black fern aphid *I. nephrelepidis* lasting for 24 hours did not provoke a quantitatively measurable reaction in terms of VOCs emission as well as of phytohormone production. When the same fern species was subjected to herbivory by a generalist leaf-chewing insect, *S. exigua* caterpillar, significant changes in the total and individual contents of VOCs emitted but not in the phytohormones after 24 hours were observed.

Although some liverwort and fern species from a same location were already shown to emit similar bulk of VOCs (Asakawa et al., 2001), we hypothesized that working with wild fern specimens, from different populations, may lead to a high variability in terms of VOCs (Effah et al., 2020, Szenteczki et al., 2021) or hormone production (Schuman et al., 2009). Interestingly, we obtained consistent results among treatments and were unable to detect differences in VOCs emissions across populations (data not shown).

In their review, Ali and Agrawal (2012) explain that phloem-feeders are frequently considered as minor plant defenses elicitors, even though much stronger effects have been documented as well. Here, our results clearly show that no significant difference between aphid-attacked and undamaged ferns can be observed (Fig. 1), strengthening the weak plant reaction to phloem-feeder hypothesis.

Ferns under threat by caterpillar herbivory, and mechanical damages to some extent, significantly changed their VOCs emissions. For instance, we measured significant increase in both most of the aldehydes (e.g. hexanal, octanal, decanal, nonanal) and many terpenes (e.g. β-pinene, limonene) after attack by caterpillar (Fig. 2 and Supp. table 2). Mechanical damages triggered a similar reaction to real herbivory, but of a smaller magnitude. Interestingly, no green leaf volatiles (GLVs), frequently observed in seed plants (Kessler and Baldwin, 2001, Dicke et al., 2009), were detected during our experiments on *P. vulgare*. In opposite, terpenoid compounds, which are well known to be released by gymno- and angiosperms under both biotic and abiotic stress, were emitted by *P. vulgare* and may suggest similar overall responsive functions in ferns. Here, quantities of six terpenoids significantly changed between undamaged and caterpillar-attacked or mechanically-damaged ferns. More β-pinene was released from both leaves attacked by caterpillar and artificially wounded, whereas higher limonene quantity was assessed in the ferns under caterpillar threat only. Those two terpenes, along with many others, were described as inducible in higher plants (Takabayashi et al., 1994), especially upon insect herbivory (Sobhy et al., 2018, Badra et al., 2021). This study shows that terpenes can be induced after biotic stress in ferns as well, at least for *P. vulgare* species (Fig. 2 and Supp. table 2), and strongly suggests the existence of chemical elicitors related to herbivory in early vascular plants. This phenomenon has been previously suggested by Radhika et al. (2012), and compared to what is seen in higher plants (Alborn et al., 1997). It is also interesting to highlight that VOCs releases in early vascular plants are not expected to influence their fertilization because they do not need to attract pollinators with floral VOCs, unlike part of the flowering plants do (Pichersky and Gershenzon, 2002). The possible use of odor cues to help the dispersal of the spores by insects or other vectors has, to our knowledge, not been investigated so far.

Phytohormones are important in plant defenses against biotic and abiotic pressures, with different hormones being involved in response to various stress types (Ku et al., 2018). They also interact with each other, using cross-talk to avoid any misallocation of the plant's resources (Walling, 2000, Bostock et al., 2001), and ultimately offer a fine-tuned, time-dependent, response to different threats. For instance, one single tomato plant adapt its responses through phytohormones, showing both higher level of JA after 72h and OPDA after 24h when attacked by a sap-sucking, compared to a leaf chewing insect (Gosset et al., 2009). We expected to see such effects in *P. vulgare*, however no significant change in phytohormonal levels was detected, no matter the herbivores tested (Fig. 3 and 4). As the levels measured in this study reflect the limited hormonal state (i.e. 6 hormones maximum) of the *P. vulgare* ferns at 24h after the beginning of attacks, we cannot rule out that other sampling timing, or plant hormones, may have given another pattern. Nevertheless, according to previous study (Radhika et al., 2012), hormones measurements after 24h make perfectly sense in term of biological relevancy. Further analyses should be carried out to properly answer this specific issue. If our results obtained from *P. vulgare* cannot allow to deeply speculate about the other fern species, changes in volatile emissions were observed among many other fern species in response to herbivory, mechanical damage or JA application. This suggest an ubiquitous targeted responses against various stresses in early vascular plants, comparable to those known in gymno- and angiosperms (Soriano and Clavijo-McCormick (2020).

When applying oral secretions of two chewing herbivores, one generalist and one specialist, or water as control, on artificially wounded ferns, Radhika et al. (2012) were not able to observe any significant difference in subsequent JA contents. However, they showed that VOCs released by *Pteridium aquilinum* fern changed under jasmonic acid application, although no correlation between frond consumption by a chewing herbivore and changes in VOCs production was found. In the present study, such effect in VOCs emitted by *Polypodium vulgare* under attack by a chewing herbivore was clearly observed (Fig. 2 and Supp. table 2).

The two main experiments were carried out on two consecutive years. Between the two years, a difference on overall VOCs released by plants is visible (Fig. 1 and 2). As all the ferns used in this study were wild plants, they may have undergone different environmental conditions and no record on their natural history was accessible. The two weeks period in controlled growth chamber before experiment and analyses certainly reduced, but not all, the likely natural differences between them. Theoretically, multiple biotic and abiotic stresses can have either an additive effect on the emission of plant VOCs, or one only leads the plant’s response (Holopainen & Gershenzon 2010). The patterns observed for the phytohormones show the opposite than for the VOCs, with higher global quantities within the first aphid vs control ferns experiment, compared to the other series of experiment (Fig. 3 and 4). We may hypothesize that wild plants from the first year suffered higher natural stresses, with higher hormonal levels influencing negatively the emissions of VOCs when subjected to an additional herbivory stress. Without any data to support this, it remains pure speculation yet.

Another concern is about the lack of VOCs change in ferns attacked by sap-sucking aphids. In many previous studies, aphids have been demonstrated to not induce plant defenses, and can sometimes even silence them (Walling, 2008). Our results on both VOCs and phytohormone show the same effect after 24 hours, with aphid-infested plants presenting similar patterns to those observed in healthy undamaged control ferns. A minority of other studies validly argue that VOCs production increase after aphid-infestation (Tun et al., 2020). As well, the question about the timing after initial infestation remains, as attack by *Idiopterus nephrelepidis* aphids may cause symptoms or systemic response later than after the 24 hours allowed in this study (Severin and Tompkins, 1950).

It is generally assumed that less VOCs are emitted in response to sap-sucking insects as compared to leaf-chewing herbivores (Turlings et al., 1998, Gosset et al., 2009). In this study, the same pattern was observed as in cotton plants attacked either by whiteflies or beet armyworms, in which the sap-sucking insect does not alter VOCs emission while the herbivory by the leaf-chewing caterpillars changed the volatiles' profile (Rodriguez-Saona et al., 2003), reinforcing the idea that conserved defensive mechanisms exist throughout the entire plants kingdom.

Especially, VOCs released by plants might be used in the third trophic level interactions, which is well known in higher plants, where an infestation by aphids induces the emission of species-specific volatile blends that are detectable by predators (Badra et al., 2021). In Malaysia, some parasitized beet armyworm (*S. exigua*) had been found on the *Shaeropteris mollucana* fern (Ghazali et al., 2014), leading to hypothesize that VOCs emitted by ferns under caterpillar's attack could be used by parasitoids. The implication of the potential phytohormonal pathways and the subsequent VOCs emissions on the interaction with the third trophic level, including predators and parasitoids that would help the ferns to defend themselves against herbivory, would definitely deserve to be properly investigated.

On the side of belowground interactions, entomopathogenic nematodes are attracted by decanal and nonanal (Laznik and Trdan, 2016), two volatile compounds which increased significantly after attack by caterpillars in our study. Those nematode worms are naturally located in the soil but current cutting-edge research aims to make them effective on plant leaves for pest management strategy like against the fall armyworms (*Spodoptera frugiperda*, Fallet et al. (2022)). It is then quite interesting to notice that they could potentially be attracted by fern VOCs as well.

6.7 Conclusion

This study provides evidences that a fern species is able to adapt its VOCs emission towards various herbivorous or artificial attacks. In that sense, we suggest here that early vascular plants may have already developed mechanisms and pathways allowing selective plant responses to biotic and abiotic stresses, well before emergence of gymno- and angiosperm species. As in the seed plants taxa, generalist chewing herbivores in ferns do not trigger the same VOCs emissions than those observed for sap-sucking attack or mechanically damaged leaves. Furthermore, the link between phytohormones production and the VOCs respond to herbivory is still unclear and need dedicated investigations, as for the possible role and importance of early land plant VOCs on the third trophic level interactions.

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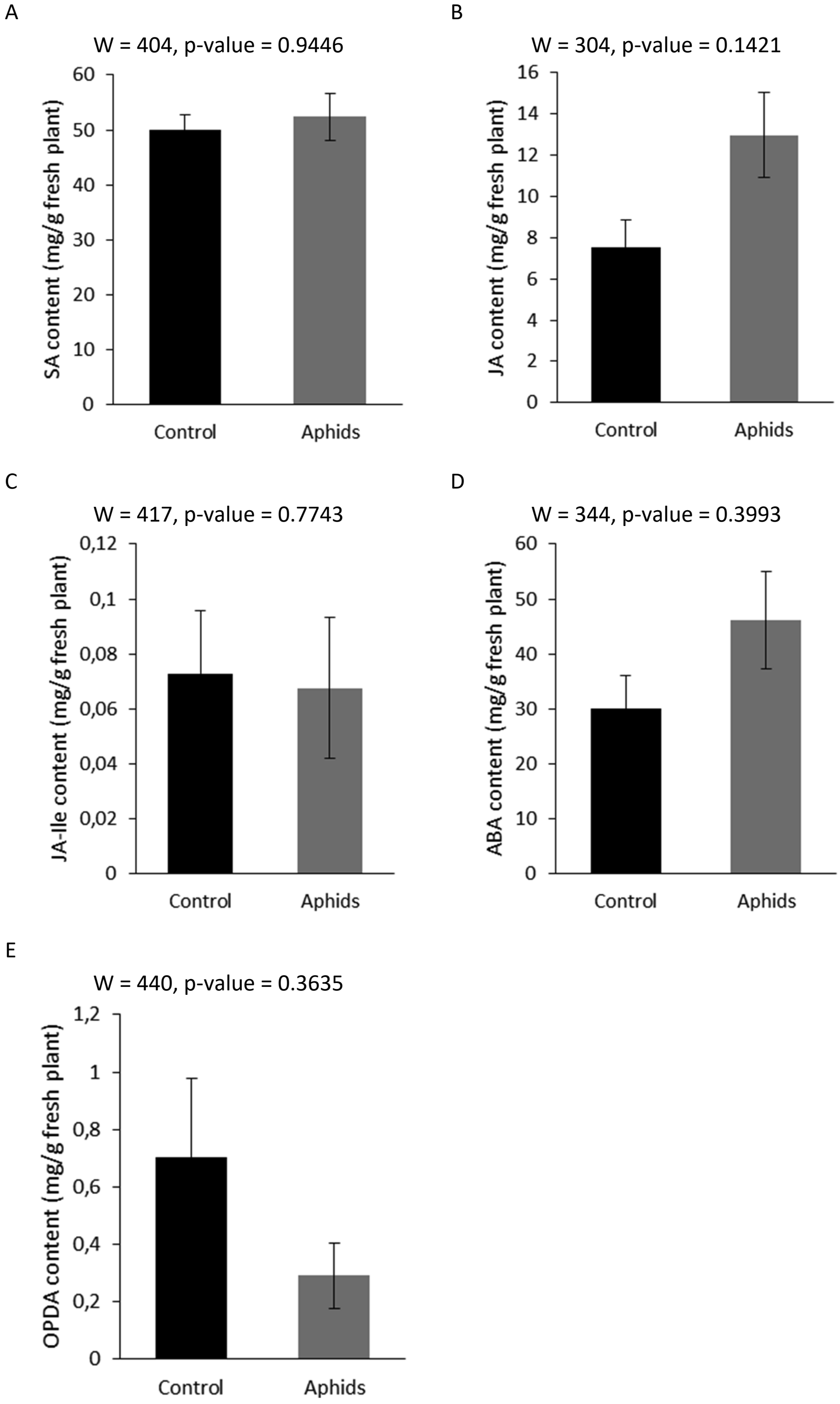
6.10 Supplementary data



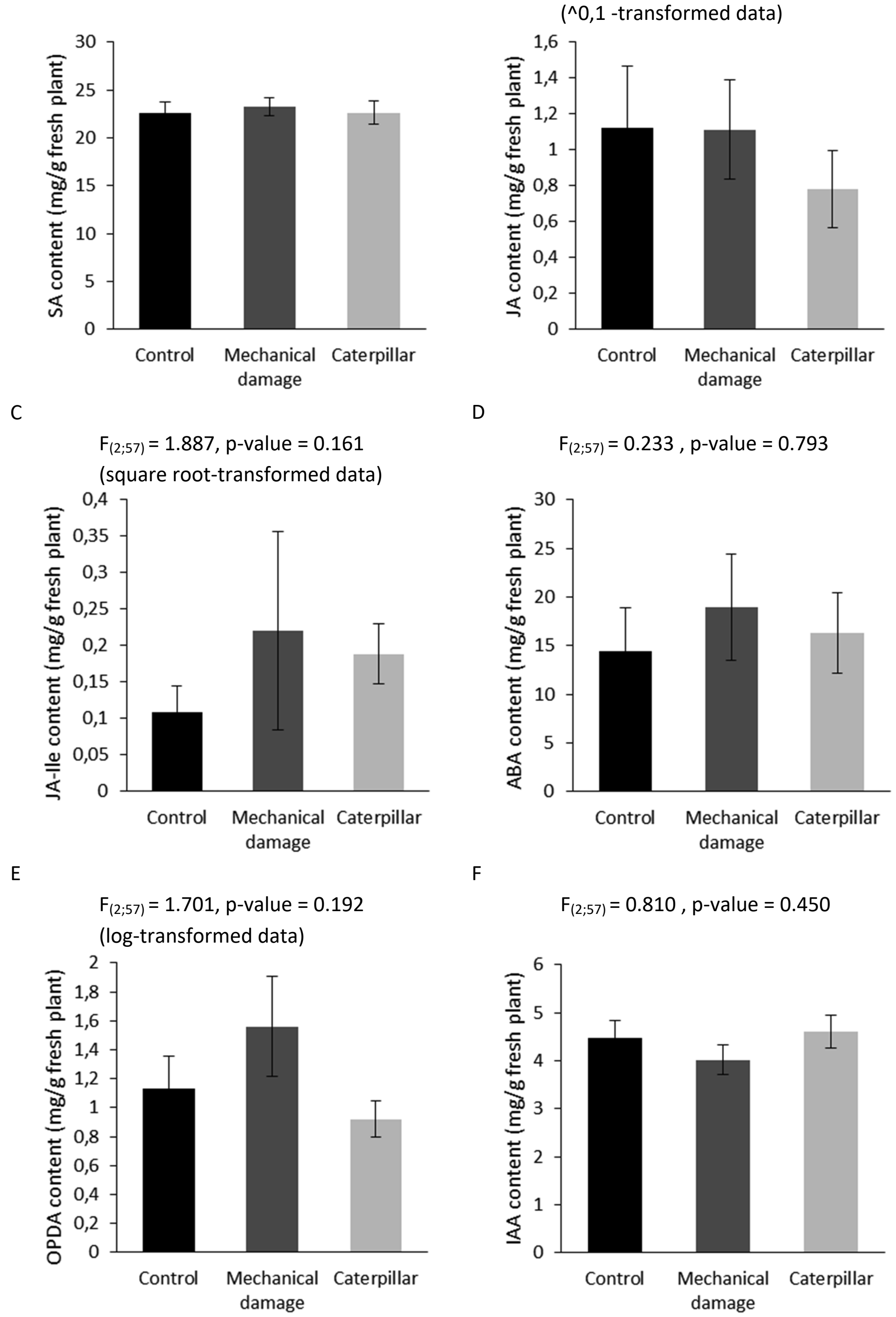
Supplementary figure 1: Individual graphs of phytohormones quantification of five different phytohormones (mg/g fresh plant) measured (mean +/- SE) in *Polypodium vulgare* ferns under the attack of *Idiopterus nephrelepidis* aphids (in gray) or not attacked as control (in black). SA = salicylic acid, JA = jasmonic acid, JA-Ile = jasmonate-isoleucin, ABA = abscisic acid, and OPDA = 12-oxophytodienoic acid.

A

$F_{(2;57)}$ = 0.125, p-value = 0.883

B

$F_{(2;57)}$ = 0.476, p-value = 0.624



Supplementary figure 2: Individual graphs of phytohormones quantification of six different phytohormones (mg/g fresh plant) measured (mean +/- SE) in *Polypodium vulgare* ferns under the attack of *Spodoptera exigua* caterpillar (in light gray), mechanically damaged (in dark gray) or not attacked as control (in black). SA = salicylic acid, JA = jasmonic acid, JA-Ile = jasmonate-isoleucin, OPDA = 12-oxophytodienoic acid, ABA = abscisic acid and IAA = Indole-3-acetic acid.

Supplementary table 1: VOCs released by *Polypodium vulgare* attacked by aphids and by undamaged control plants. The columns show tentative identity of the compound, related retention time (RT), chemical class, mean quantity (± SE), results of Wilcoxon's test (W and p-values), respectively.

Compound	RT	Class	Mean (± SE) Aphids	Mean (± SE) Control	W	p-value
Hexanal	3.1	Aldehyde	1.160 (±0.233)	1.597 (±0.334)	454.5	0.143
2-Pentanone, 4-hydroxy-4-methyl	3.82	Ketone	1.220 (±0.329)	1.485 (±0.378)	421.0	0.059
3-Decyn-2-ol	4.45	Alcohol	0.331 (±0.095)	0.397 (±0.135)	531.5	0.531
Heptanal	4.73	Aldehyde	1.373 (±0.303)	0.837 (±0.248)	635.0	0.473
L-(-)-Menthol	5.11	Alcohol	0.107 (±0.023)	0.107 (±0.033)	560.5	0.841
alpha-Thujene	5.27	Monoterpene	0.071 (±0.021)	0.088 (±0.037)	570.0	0.931
alpha-Pinene	5.43	Monoterpene	0.573 (±0.117)	1.103 (±0.368)	487.0	0.282
2-Heptanone, 4-methyl-	5.54	Ketone	0.841 (±0.272)	0.746 (±0.210)	489.0	0.235
beta-Pinene	6.4	Monoterpene	1.751 (±0.547)	2.293 (±0.633)	505.5	0.371
2H-Pyran-2-one, tetrahydro-6-pentyl-	6.67	Lactone	1.214 (±0.300)	1.462 (±0.439)	516.0	0.455
2(3H)-Furanone, dihydro-3,5-dimethyl	6.83	Lactone	2.795 (±0.619)	1.814 (±0.459)	604.0	0.739
Octanal	7.05	Aldehyde	2.977 (±0.328)	2.627 (±0.372)	610.0	0.691
Nonatriene	7.36	Terpene	0.615 (±0.173)	0.708 (±0.232)	482.0	0.240
3-Hexene-2,5-diol	7.42	Alcohol	0.672 (±0.187)	0.703 (±0.271)	564.0	0.885
1,3,8-p-Menthatriene	7.58	Monoterpene	0.737 (±0.413)	5.486 (±5.289)	472.5	0.201
D-Limonene	7.69	Monoterpene	8.253 (±3.196)	3.956 (±0.980)	548.0	0.744
Isogeraniol	7.88	Aliphatic alcohol	0.744 (±0.164)	1.531 (±0.846)	567.5	0.922
2-Undecanone, 6,10-dimethyl-	7.99	Ketone	3.579 (±0.517)	3.579 (±0.627)	539.0	0.665
2-Hexyl-1-octanol	9.00	Aliphatic alcohol	1.186 (±0.179)	1.793 (±0.440)	503.0	0.386
Nonanal	9.69	Aldehyde	13.021 (±1.636)	13.987 (±2.037)	488.0	0.298
Isomenthol acetate	9.96	Monoterpene	8.254 (±1.003)	7.638 (±1.116)	557.0	0.826
cis-p-Menth-2,8-dienol	10.13	Monoterpene	0.823 (±0.184)	4.613 (±4.089)	509.0	0.426
6-Octen-1-ol, 3,7-dimethyl-	11.48	Monoterpene	1.717 (±0.320)	1.861 (±0.469)	535.0	0.628
Decanal	12.44	Aldehyde	7.790 (±1.257)	7.278 (±1.147)	554.0	0.799
Pinocarvone	13.06	Monoterpene	0.607 (±0.163)	0.388 (±0.089)	555.0	0.797
(+)-Carvone	13.48	Monoterpene	0.535 (±0.079)	0.468 (±0.072)	591.0	0.862
p-Menthane-1,2,3-triol	13.93	Monoterpene	1.442 (±0.242)	1.459 (±0.370)	581.0	0.957
δ-Guaiene	18.64	Sesquiterpene	3.347 (±0.333)	2.815 (±0.379)	655.0	0.348
cis-α-Bisabolene	21.07	Sesquiterpene	0.348 (±0.079)	0.500 (±0.246)	551.5	0.773
Total VOCs			68.087 (±8.070)	73.318 (±14.543)		

Supplementary table 2: VOCs measured (mean (± SE)) on the fern *Polypodium vulgare* attacked by aphids or undamaged (control), related retention time (RT) and chemical class the compound belongs to (Class), with the results of Kruskal-Wallis tests (Chi^2) and p-values associated.

Compound	**RT**	**Class**	**Mean (± SE) Caterpillar**	**Mean (± SE) Mechanical damage**	**Mean (± SE) Control**	**Chi^2**	**p-value**
Acetaldehyde	1.49	Aldehyde	2.419 (±0.420)	2.404 (±0.393)	2.337 (±0.527)	0.311	0.856
Butanal, 3-methyl-	2.37	Aldehyde	5.126 (±0.230)	3.021 (±0.435)	2.071 (±0.313)	2.980	0.225
Hexanal	3.32	Aldehyde	3.540 (±0.522)	6.883 (±0.223)	2.992 (±0.380)	12.372	0.002*
2-Pentanone, 4-hydroxy-4-methyl-	3.92	Ketone	4.062 (±0.593)	5.623 (±0.610)	3.021 (±0.795)	0.685	0.710
5,9-Dodecadien-2-one, 6,10-dimethyl-, (E,E))-	4.16	Ketone	2.780 (±0.433)	2.681 (±0.319)	1.960 (±0.326)	3.049	0.218
3-Decyn-2-ol	4.55	Alcohol	1.282 (±0.252)	1.848 (±0.290)	1.393 (±0.350)	4.213	0.122
Heptanal	4.94	Aldehyde	5.479 (±0.461)	4.207 (±0.754)	3.644 (±0.956)	1.345	0.511
L-(-)-Menthol	5.22	Alcohol	1.927 (±0.513)	1.438 (±0.203)	1.615 (±0.359)	0.322	0.851
alpha-Thujene	5.27	Monoterpene	1.334 (±0.331)	1.283 (±0.201)	1.159 (±0.220)	0.608	0.738
α-Pinene	5.48	Monoterpene	2.093 (±1.587)	1.282 (±1.219)	2.606 (±1.256)	0.825	0.662
2-Heptanone, 4-methyl-	5.7	Alcohol	4.633 (±1.220)	5.718 (±1.097)	2.741 (±1.074)	1.180	0.555
Benzaldehyde	6.2	Aldehyde	9.100 (±1.235)	11.938 (±1.333)	7.337 (±1.234)	3.679	0.159
β-Pinene	6.62	Monoterpene	25.923 (±1.263)	45.049 (±1.013)	7.034 (±1.762)	32.700	< 0.001*
2H-Pyran-2-one, tetrahydro-6-pentyl-	6.9	Lactone	5.471 (±0.385)	7.652 (±0.748)	3.184 (±0.594)	4.468	0.107
2(3H)-Furanone, dihydro-3,5-dimethyl	6.98	Lactone	11.323 (±1.807)	6.188 (±1.232)	5.679 (±1.082)	1.890	0.389
Octanal	7.19	Aldehyde	6.599 (±2.996)	18.636 (±2.360)	7.329 (±2.132)	24.012	< 0.001*
Nonatriene	7.49	Terpene	3.956 (±0.379)	3.834 (±0.887)	2.607 (±0.615)	1.800	0.407
3-Hexene-2,5-diol	7.56	Alcohol	3.488 (±0.349)	2.745 (±0.712)	2.131 (±0.586)	0.602	0.740
1,3,8-p-Menthatriene	7.74	Monoterpene	0.985 (±0.276)	2.006 (±0.849)	0.989 (±0.475)	7.034	0.030*
D-Limonene	7.82	Monoterpene	6.881 (±4.348)	13.522 (±4.187)	9.646 (±4.595)	13.225	0.001*
Isogeraniol	7.88	Aliphatic alcohol	2.416 (±0.439)	5.456 (±0.946)	2.108 (±0.357)	13.815	0.001*
Benzeneacetaldehyde	8.22	Aldehyde	2.524 (±1.585)	3.486 (±1.509)	2.739 (±1.165)	11.341	0.003*
2-Octenal, (E)-	8.59	Aldehyde	2.087 (±0.310)	1.982 (±0.357)	1.677 (±0.409)	3.460	0.177
2-Hexyl-1-octanol	9.12	Aliphatic alcohol	4.308 (±0.498)	4.282 (±0.111)	2.848 (±0.597)	0.659	0.719
Undecane	9.7	Alkane	1.842 (±0.272)	2.379 (±0.439)	1.826 (±0.432)	2.505	0.286
Nonanal	9.82	Aldehyde	17.343 (±1.411)	64.907 (±1.497)	13.959 (±1.875)	29.913	< 0.001*
Isomenthol acetate	10.1	Monoterpene	12.039 (±1.942)	9.968 (±1.568)	7.246 (±1.865)	3.378	0.185
cis-p-Menth-2,8-dienol	10.73	Monoterpene	1.561 (±0.576)	2.193 (±0.700)	1.697 (±0.907)	2.599	0.273
2-Nonenal, (E)-	11.31	Aldehyde	7.684 (±0.734)	7.275 (±0.744)	2.869 (±0.400)	20.335	< 0.001*
6-Octen-1-ol, 3,7-dimethyl-	12.12	Monoterpene	2.296 (±0.387)	10.317 (±0.303)	2.667 (±0.681)	10.256	0.006*
Dodecane	12.41	Alkane	2.459 (±0.582)	3.672 (±0.670)	1.787 (±0.364)	6.581	0.037*
Decanal	12.6	Aldehyde	14.720 (±2.369)	81.216 (±2.959)	11.951 (±2.046)	33.008	< 0.001*
Pinocarvone	13.03	Monoterpene	2.493 (±0.338)	4.279 (±0.580)	2.522 (±0.581)	8.519	0.014*

Supplementary table 2: continued.

Compound	RT	Class	Mean (± SE) Caterpillar	Mean (± SE) Mechanical damage	Mean (± SE) Control	Chi^2	p-value
Geranyl isovalerate	13.63	Terpene	1.887 (±0.353)	4.348 (±0.929)	2.045 (±0.719)	7.173	0.028*
p-Menthane-1,2,3-triol	13.93	Monoterpene	1.915 (±0.379)	3.398 (±0.535)	1.318 (±0.186)	12.553	0.002*
2-Decenal, (E)-	14.07	Aldehyde	1.424 (±0.203)	2.904 (±0.944)	1.717 (±0.293)	0.573	0.751
Nonanoic acid	14.35	Fatty acid	2.540 (±3.439)	6.961 (±3.744)	4.894 (±3.525)	8.314	0.016*
Tridecane	15.08	Alkane	17.666 (±1.586)	11.927 (±1.753)	12.095 (±1.884)	1.016	0.602
Undecanal	15.28	Aldehyde	5.609 (±1.319)	14.076 (±1.312)	5.422 (±1.192)	13.933	< 0.001*
5-Octen-2-one, 3,6-dimethyl-	16.46	Ketone	3.505 (±0.286)	2.799 (±0.063)	1.996 (±0.313)	1.257	0.533
n-Decanoic acid	16.84	Fatty acid	2.434 (±1.620)	7.247 (±1.662)	3.025 (±1.320)	11.676	0.003*
Propanoic acid, 2-methyl-, 3-hydroxy-2,4,4-trimethylpentyl ester	17.01	Carboxylic ester	3.844 (±1.781)	7.466 (±1.260)	2.963 (±1.188)	13.690	0.001*
Tetradecane	17.67	Alkane	1.856 (±0.629)	13.211 (±0.960)	1.502 (±0.478)	25.443	< 0.001*
Dodecanal	17.89	Aldehyde	2.063 (±0.414)	12.261 (±0.411)	1.913 (±0.385)	27.228	< 0.001*
δ-Guaiene	18.75	Sesquiterpene	5.146 (±0.631)	3.940 (±0.497)	3.948 (±0.514)	2.130	0.345
Neocurdione	19.47	Ketone	21.792 (±3.090)	30.837 (±3.550)	19.221 (±3.335)	4.379	0.112
2,2,7,7-Tetramethyltricyclo[6.2.1.0(1,6)]undec-4-en-3-one	19.66	Ketone	2.153 (±4.665)	6.478 (±4.243)	6.100 (±4.112)	9.720	0.008*
Pentadecane	20.13	Alkane	2.769 (±0.157)	7.766 (±0.309)	2.256 (±0.850)	21.629	< 0.001*
Hexadecane	22.46	Alkane	5.956 (±1.301)	12.643 (±1.890)	4.782 (±1.817)	11.007	0.004*
Benzophenone	23.09	Ketone	2.474 (±0.642)	8.314 (±0.516)	2.049 (±0.448)	19.888	< 0.001*
n-Hexadecanoic acid	30.04	Fatty acid	31.419 (±11.420)	39.760 (±11.484)	24.395 (±11.859)	6.514	0.039*
Total VOCs			545.706 (±70.934)	298.623 (±42.258)	229.013 (±41.336)		

7 General discussion and conclusion

Herbivory is a common phenomenon among plants and quite well studied among angiosperm species, which gathers the higher plant linages from an evolutionary point of view (Marquis, 1992). The early land plants which are bryophytes *sensu lato* and the first vascular plant group, the pteridophytes (Gerson, 1969, Glime, 2006), are less studied in general, and about this subject in particular (Ranker, 2016, Hodson et al., 2005, Markham et al., 2006, Atherton et al., 2010). Aiming to fill this gap of knowledge, I attempted to study several aspects of the interaction between those primitive plant groups and the potent herbivores they encounter in their environment. In plants, the defense strategy is theoretically based on a triangle between: (1) tolerance/escape; (2) low nutritional quality and; (3) nutrition and defense (Agrawal and Fishbein, 2006). I consequently focused my researches on the nutritive and defensive aspects of the interaction of herbivory taking place between early land plants and phytophagous organisms. I included up to 15 lower plant linages species and different species of invertebrate herbivores, from different taxonomic groups (*i.e.* one generalist mollusk, one generalist leaf-chewing insect, and one fern-specialized sap-sucking insect).

In the first chapter, I focused on a common assumption which state that bryophytes and pteridophytes do not exhibit a sustainable level of nutrients for herbivores (Ehrlich and Raven, 1964, Hernick et al., 2008, Prins, 1982). The nutrients are primary metabolites and, as their name state are primary, so we hypothesize that plants as these plants should not be as poor because they possess those nutrients for their own metabolism in the first place and not only to keep them available for herbivores. I then demonstrated that the levels and ratios are not problematic, suggesting that another factor was responsible for the bad performances of the herbivores feeding upon them (Duhin et al., 2022).

Even though the proteins were only studied as nutrients and were not specifically identified in my study, some of them can have a defensive role in plants. This aspect had been studied by Markham et al. (2006), who clearly demonstrated that it happens in primitive plants like mosses and ferns. When I used artificial diet mixed with plant powder in the second chapter, I record a striking deference in the performances of the *S. exigua* caterpillars on mosses diet. They were able to develop and become adult as good and fast as on higher plant mixed-diet used as control. In the diet preparation process, the powders were added at around 80°C, and this temperature is high enough to denature the covalent bounds maintaining proteins folded and able to fulfill the function of the proteins (Somero, 1995). That would of course include the defensive role if the studied mosses have their strategy based on that. Alternatively, I would hypothesize that the deference in the performances of the *S. exigua* caterpillars on mosses diet between the first or second chapter of this thesis suggest that there is a structural problem for the generalist herbivore that was used (*S. exigua*), or for herbivores in general, with the fact to feed on bryophytes, as it is a general trend for small plant species to have lower herbivore richness (Haysom and Coulson, 1998, Schlinkert et al., 2015). There could be a pressure to avoid this food source as it is composed of small leaves that would imply to move from one to another every two bites and it would result in an unfavorable cost-benefit ratio for the herbivore.

It also interesting to notice that the proteins have also been studied in ferns as a protection against sap-sucking insects (Shukla et al., 2016). This mechanism could explain why I was not able to record any change in the VOCs profile of the ferns submitted to an aphid attack in the fourth chapter. Indeed, if this mechanism is common among fern species, there is no need and no reason to invest in another one like VOCs. This could explain my findings on the lack of apparent VOCs difference between control and aphids-attacked plants. Furthermore, we can find a link between proteins and VOCs emission in plants. Indeed, the quality of the food in terms of high protein content can be advertised by VOCs, and bumblebees are able to discriminate between food sources thanks to VOCs emissions (Rasheed and HARDER, 1997). However, it could be specific to this type of herbivore who collect food source for offspring and not for their own consumption and then, should recognize the quality of the food while foraging and without ingesting it. Nevertheless, the investigation of the correlation between protein content and VOCs would be an interesting further step to study in the context of herbivory on primitive plants.

There is also a role played by water in the protein dynamic (Tarek and Tobias, 2002) that could partially connect the relatively high protein content found in the fern species investigated in the first chapter and their water content measured in the second one. Furthermore, water has very interesting link with another primary metabolite class which are the sugars. Indeed, those are used in the desiccation tolerance mechanisms in seed of higher plants (Koster and Leopold, 1988), and which is particularly efficient in the bryophyte species (Proctor et al., 2007). I found in the first chapter a relatively high content in sugar in the moss species and high water in chapter 2 also in these plants and those two pieces of information come along with each other to corroborate and reinforce the high tolerance upon desiccation theory in bryophytes. Moreover, mosses are a changing resource in terms of water content particularly because they can lose and regain their aqueous content fast and efficiently due to their ability to tolerant desiccation (Oliver et al., 2005). Therefore, this resource would not be reliable for short life-time organisms like herbivorous insects. Another particularity of the groups of early land plants investigated during this thesis, is that they have recourse to alternate sporophyte/gametophyte phases, and link to that, use spores in their sexual reproductive cycle, and this one relies on water, unlike angiosperms' one (Renzaglia et al., 2000). Those elements taken together show the importance of water for the lower plants and its connection with herbivory.

The physical defenses in early land plant linages are an interesting subject that I investigated in the second chapter. Nevertheless, the number of species was limited and only one species presented macroscopic trichomes. We definitely do not know everything about those hairy structures in primitive plants as Rodriguez et al. (2018) found out that one species of fern exhibit trichomes on the adaxial surface in young fronds that disappears on mature leaves, even though the ferns' trichomes are generally abaxial. These structural defenses have been proven to have bioactive secretions against fungi and herbivorous mollusks (Rodriguez et al., 2018). This shed the light on the fact that when talking about trichomes, the frontier between their mechanical defensive role and their chemically-inducible defensive function when we face glandular trichomes (Wagner, 1991). Indeed, an important part of the defense strategy in plants relies on inducible defenses (Tollrian and Harvell, 1999). It is the case for the physical defenses, the chemical ones present inside the cells of the plants as well as the VOCs released in the neighboring environment. Therefore, I would hypothesize that the early land plants would also use this mechanism and perhaps that this one has an old origin, and is shared by all the embryophytes.

The other physical defense that I investigated in the second chapter was the silica present in plant's leaves. This defense is also inducible upon stress including herbivory, via the phytohormones pathways (Johnson et al., 2021, Hartley and DeGabriel, 2016). The silica can present several forms in the plant and one of those is needles, which are used to inject pathogen in the body of the herbivore consuming the tissues of the plant (Lev-Yadun and Halpern, 2019). This phenomenon has also been described for bigger structures like thorn can (Halpern et al., 2007).

The herbivory undertaken by aerial parts of the plants are known to have an effect on the soil biota occurring belowground (Bardgett and Wardle, 2003). When discussion this topic, it is important to note that mosses do not possess proper roots or rhizomes, like higher plants dot, but only rhizoids, which are hair-like structures used by those plants to stay attached to their subtract and also draw water (Proctor, 2000, Odu, 1978). Very interestingly, these structures are analogous to the hairs present on the root system of higher plants (Jones and Dolan, 2012). Therefore, it would be interesting to investigate those as a food source for small belowground herbivores and compare the performances of those on different group of plants. Of course, the establishment of the experimental design is very challenging as, if we want to compare strictly what is comparable (*i.e.* only the hairy structures from both mosses and higher plants), we should prevent the consumption of the main part of the root or rhizome of the plant and only allow the access to the hairs. Investigations have predominantly focused on the foliar apparatus of the plants, but belowground interactions are also occurring with the true roots and rhizomes in ferns, even though the studies in that context are very rare, but the few carried out have shown that herbivory can kill root system of ferns (Rayamajhi et al., 2014). It would then be very interesting to investigate, like it has been done in higher plants, above- vs. belowground attack separately or simultaneously, which is scenario likely to append under natural conditions (Dicke et al., 2009).

After addressing belowground interactions, the logical further step would be to study the case of a multiple attack and its consequences in terms of early land plants' defensive response. I would then hypothesize that we would observe the same pattern as for higher plants lineage, meaning an alteration of the capacity to cope with both treats leading to an intermediate level of defense (Rasmann and Turlings, 2007). Multiple attack can also be investigated on the same part of the plant with multiple attackers. For instance, this case with pierce-sucking insects can result in the production of an increased release of VOCs, compared to single herbivory and subsequently attract a predator more efficiently, providing better protection for the plant (Moayeri et al., 2007).

Even though the question was not investigated in this thesis, the influence of the microorganisms' interaction with the plants and the herbivores is an interesting topic. I believe that this could be an important part of the understanding of the primitive plants and their interactions in the environment. Indeed, the microorganisms present in the gut of the phytophagous insects can participate to the detoxification of the food ingested (Hammer and Bowers, 2015, Wielkopolan and Obrępalska-Stęplowska, 2016, Acevedo et al., 2017). On the other hand, there could also be toxic effect of the plants on the gut flora of the herbivores through the use of protease inhibitors, which have a negative impact on the nutrient assimilation (Zhu-Salzman and Zeng, 2015). We can therefore imagine that primitive plants can employ this mechanism as well.

Mastering the interactions with microorganisms could also be the key of the good resistance of ferns to sap-sucking insects, which are most of the time not causing damages by themselves, but rather because they transmit viral or bacterial diseases (Ng and Perry, 2004, Eastop, 1977). Mosses on the other hand, have no vascular system and then are logically not a sustainable host for the sap-sucking herbivores, but piercer organisms are nevertheless reported on mosses, like the moss bug and the tardigrades (Moran et al., 2005, Schill et al., 2011). The studies on those organisms and their interaction with their host plants are really scares. Regarding transmission of pathogens, both of the primitive plant groups I have studied here (*i.e.* bryophytes and pteridophytes) have evolved a good resistance to fungal pathogens (Mewari and Kumar, 2011). Concerning the ferns, it has been shown that they are significantly less attacked by fungi than angiosperms (Antonovics, 2020).

When talking about early land plants, there is an evolutional perspective to take into account. For instance, I would like to highlight the fact that ferns like *Dryopteris filix-mas*, on which I focused in the third chapter of my thesis, possess similar defenses compounds (*i.e.* the phloroglucinols) as the soil bacteria *Pseudomonas fluorescens*. Moreover, the study of Shukla et al. (2016) focuses on GMOs crops made with fern genes. This is a highly interesting area of study, aiming to study and use more early land plants defensive mechanisms in crop defense. This idea is already concretized with bacterial phloroglucinols genes (Fenton et al., 1992, Dowling and O'Gara, 1994, Abdel-Ghany et al., 2016) and it would be interesting to investigate as a further perspective to my study. Nevertheless, to get to this point, the information of the genetic pathways of the expression of the phloroglucinols in the *Dryopteris* ferns.

Regarding this evolutionary perspective still, we must address the topic of early interactions with ancient herbivores. Mosses are quite hard to study in that regard because the softness of their tissues is not favorable to fossilization (Tomescu et al., 2018). On the other hand, there are insects' traces of feeding on ferns aging from latest Silurian to Early Devonian (417 to 403 Ma) (Labandeira, 2007). These groups of plants have also coexisted with another major group of herbivores which are the dinosaurs. Of course, we cannot today verify the theories that are elaborated about the interactions between the plants and these animals, but they have happened and their ecological consequences have been studied (Hummel et al., 2008). The ferns are then likely to have evolved defensive strategies against the large organisms that the dinosaurs are and we are perhaps witnessing the remains of this defenses through the toxic chemical compounds found in the pteridophytes nowadays. Moreover, the specialization of the insects on the pteridophytes are nowadays viewed as a parallel occurrence as the one that happened between insects and angiosperms. For instance, the fern-sawfly interaction appeared in the Cretaceous era (145 to 66 Ma) (Schneider, 2016).

The early land plants are interesting models to study the evolution of various mechanisms in plants. Nevertheless, the fact that many researches keep thinking of those plants and referring to them as "primitive" is counterproductive in the study field and does not help filling the gap of knowledge about the early land plants(Watkins Jr et al., 2007). After all, those plants are currently living in various ecosystems, and with the same constraints as any other higher plant on Earth. Moreover, they successful and adapted to their environment, and that implies that they can manage abiotic and biotic stress, including herbivory, thanks to their long evolution.

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