DEPARTAMENTO DE CIÊNCIAS DA VIDA
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Sexual segregation in red deer: a question of food?

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Ecologia, realizada sob orientação da Doutora Joana Alves (Centro de Ecologia Funcional) e do Professor Doutor José Paulo Sousa (Departamento de Ciências da Vida, Universidade de Coimbra)

Fernanda Fernandes Garcia

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Resumo

A segregação sexual é um fenómeno muito comum em vertebrados, em particular em animais com dimorfismo sexual, e pode ter implicações na sua gestão. Em termos de definição, é ainda difícil encontrar consenso sobre o que é a segregação sexual, pelo que existem muitas questões ainda sem resposta. A segregação sexual pode ser explicada através de dois componentes 1) segregação de habitat ou 2) segregação social. Na tentativa de tentar entender quais são os fatores envolvidos neste fenómeno, ambos os componentes devem ser avaliados. Este trabalho tem como objetivo avaliar o componente do habitat, avaliando uma das hipóteses que foi proposta para explica-lo, a hipótese da seletividade alimentar (FSH). Amostras de fezes de veado foram recolhidas por 1) observação direta na área de estudo Serra da Lousã, ou 2) em animais abatidos em montarias. As amostras de fezes foram analisadas usando a técnica microhistólogica. Esta técnica baseia-se na identificação dos fragmentos de plantas presentes nas fezes, e é constituída por duas fases 1) elaboração da coleção de referência de epidermes e 2) identificação dos fragmentos de plantas presentes nas fezes. A coleção de referência das epidermes, juntamente com a chave dicotómica de identificação com base em características micro-histológicas, provou ser uma ferramenta útil neste tipo de análise. Embora a técnica micro-histológica tenha bastantes vantagens, tem também a desvantagem do tempo requerido para identificar os fragmentos. De forma a colmatar esta desvantagem, é fulcral determinar o esforço de amostragem adequado aos objetivos específicos de cada trabalho, de forma a otimizar o tempo. Os resultados mostraram que um total de 200 fragmentos constitui um bom compromisso entre precisão e custo, permitindo contabilizar 95% da riqueza específica.

O principal objetivo desta tese foi avaliar o comportamento alimentar de veados, e avaliar se as diferenças entre os sexos são suficientes para suportar a FSH. A dieta veado foi analisada em termos de composição, diversidade e qualidade. Os resultados obtidos não suportam na totalidade os pressupostos da FSH, principalmente os relacionados com a qualidade alimentar. Machos e fêmeas têm diferentes necessidades nutricionais, contudo, os nossos resultados não permitem concluir que os machos consomem mais quantidade e menor qualidade alimentar, e que as fêmeas selecionam habitats de alta qualidade e/ou maior qualidade de recursos alimentares. Os resultados deste estudo não provaram a existência de diferenças entre qualidade da dieta de ambos os sexos, avaliada por análises de pigmentos fotossintéticos (carotenoides e as clorofilas) presentes nas fezes. Concluindo, existem outros fatores, além do comportamento alimentar, que poderão estar na origem da segregação sexual.

Palavras chave: *Cervus elaphus,* segregação sexual, hipótese da seletividade alimentar, comportamento alimentar

Abstract

Sexual segregation is a widespread and common phenomenon among vertebrates and dimorphic animals having implications in their management. No completely agreement on the definition of this phenomenon has been achieved, and there are yet numerous unanswered questions. However, it is known that sexual segregation can be explained by either two components 1) habitat/spatial segregation or 2) social segregation. To try to understand which are the factors behind this phenomenon, it is essential to evaluate both components. Our study aims to evaluate the component of habitat segregation by testing one of the hypotheses that have been postulated to explain it, the forage selection hypotheses (FSH). We collected samples of faeces from red deer by 1) direct observations in the study area Lousã Mountain or 2) by hunted animals. We analysed those faecal samples by applying the microhistological technique. This technique is based on identification of plant fragments present in the faeces and is divided in two phases 1) construction of a reference collection of epidermis and 2) identification of plant fragments present in the faeces. The reference collection of epidermis together with the dichotomous key of identification based on microhistological features, proved to be a useful tool in microhistologic analyses. Although microhistological technique has some advantages, it has also the disadvantage of the time needed to analyse the fragments. To overcome this shortcoming, assess the optimal sampling effort, adequate to the objectives of each study, proved to be essential and time saving. Our analyses of sampling effort showed that with a total of 200 analysed fragments, a good relation between precision and cost is obtained, allowing to achieve 95% of species richness.

The major aim of this thesis was to assess the feeding behaviour of red deer, and evaluate if the differences between sexes are enough to support FSH. Red deer diet was analysed in terms of composition, diversity and quality. Our results do not fully support the predictions of FSH, mainly the prediction related with quality of forage. Males and females have different nutritional needs, however our results do not conclude that males consume more quantity and lower quality food items, while females select high quality habitats and higher food quality. The results of this study do not found significant differences between quality of diet of both sexes, assessed by analyses of photosynthetic pigments (carotenoids and chlorophylls) present in the faeces. We are able to conclude that other factors besides foraging ecology could be behind this sexual segregation.

Key words: *Cervus elaphus,* sexual segregation, forage selection hypothesis, feeding behaviour

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Chapter I - General introduction

1.1) Sexual segregation

Sexual segregation is the term that describes social or habitat segregation between males and females. Actually, sexual segregation is the separation between males and females either by social, habitat or spatial factors outside the breeding season. It is very common among vertebrates and other dimorphic animals (Mysterud 2000; Bowyer 2004), being especially pronounced among the Cervidae family (Bowyer et al. 2002). However, it can also happen in non-dimorphic species such as whales, seals, monkeys, elephants, fish and birds (Ruckstuhl & Neuhaus 2000).

Sexual segregation can be described by habitat/spatial or social segregation between males and females, *i.e.* the sexes are separated either by different habitat use (Kie & Bowyer 1999) or into different groups (Conradt 1998). These components may influence sexual segregation independently (Conradt & Roper 2005) or together. It is essential to evaluate how much habitat and space or social factors may influence sexual segregation, to understand the patterns of this phenomenon (Alves et al. 2013). Many factors could be involved in sexual segregation because males and females have differences in energetic requirements, reproductive strategies, anti-predator avoidance strategies, social affinities and activity budgets (Ruckstuhl & Neuhaus 2005; Clutton-Brock et al. 1982). To explain sexual segregation, many hypotheses are put forward to elucidate both components: habitat/spatial and social (Ruckstuhl & Neuhaus 2000; Ruckstuhl & Neuhaus 2002; Bowyer 2004; Conradt et al. 2001; Bonenfant et al. 2004; Ruckstuhl & Neuhaus 2005).

Habitat segregation, sometimes also known as ecological segregation (Mysterud 2000; Ruckstuhl & Neuhaus 2005), suggests differences between animal classes in their spatial distribution, enabling them to use different physical environments (Conradt 1998). According to Conradt et al. (1999) and Ruckstuhl & Neuhaus (2005), habitat segregation should be always studied with spatial and social segregation. To enlighten the habitat/spatial component, two main hypotheses have been postulated, the forage selection hypothesis (FSH) (also known as sexual dimorphism body size, gastrocentric or nutritional need hypothesis) and the reproductive strategy hypothesis (RSH) (also known as predation-risk hypothesis) (Ruckstuhl & Neuhaus 2005). These two hypotheses are based on the sexual body-size dimorphism (Ruckstuhl & Neuhaus 2000; Ruckstuhl & Neuhaus 2002).

The FSH predicts that sexual body-size dimorphism causes differences in sex-specific nutritional requirements that are related to food selection and subsequently habitat segregation (Ruckstuhl

& Neuhaus 2000). However, in accordance with this hypothesis, in non-dimorphic species, males and females (except lactating females) might select the same food quality (Ruckstuhl & Neuhaus 2002), which based on FSH lead them without reason to segregate. This hypothesis was initially supported by the Jarman-Bell principle (Jarman 1974) that predicts that larger body herbivores (males), will consume more ubiquitous and fibrous plants than the smaller ones (females). This principle is related to the gut capacity that increases proportionately with body mass, whereas metabolic requirements increases allometrically with body mass (Demment & Van Soest 1985). Indeed, Barboza & Bowyer (2000), suggested a gastrocentric hypothesis in which high-fibre forages will be consumed by males because their ruminal capacity prolongs retention, allowing a greater use of fibres for energy. As a result of this adaptation, males might prefer habitats with lower quality forage but higher forage biomass, because they are capable of digesting these food resources due to their larger rumen and slow passage rate of food. Whilst, females might choose habitats with lower quantity but high-quality forage (high nitrogen and low fibre levels) because they are less efficient at digesting fibrous plants owing to a small stomach size and low gut capacity (Bowyer 2004). Even within females, non-lactating females might segregate from lactating females because of their different nutritional needs (Bowyer 1984; Alves et al. 2013). Nevertheless, forage selection hypothesis is controversial (Ruckstuhl & Neuhaus 2000) because it assumes that males will select abundant low-quality forage even if high-quality forage is available (Main et al. 1996).

The RSH is based on the different reproductive strategies of males and females, which are influenced by different selective pressures (Bowyer 2004; Alves et al. 2013; Ruckstuhl & Neuhaus 2005). This hypothesis takes into account that females with young are more susceptible and vulnerable to predators than adult males (Ruckstuhl & Neuhaus 2000; Ruckstuhl & Neuhaus 2002; Ruckstuhl & Neuhaus 2005). This difference in predation pressure could lead to habitat segregation between the sexes, especially if one habitat provides more protection than the other (Ruckstuhl & Neuhaus 2000; Ruckstuhl & Neuhaus 2002; Ruckstuhl & Neuhaus 2005). Because the reproductive success of females depends on the survival of calf, they will choose safer places even independently of the quality of forage, at least whilst the offspring is not old enough to self-protection (Main & Coblentz 1996). On the other hand, the reproductive success of males depends on their physical condition, which is related to the accumulation of energy reserves for the mating season and also to compete with other males. Thus, males are likely to choose habitats with high quality of food (Ruckstuhl & Neuhaus 2005).

Sex differences in habitat use due to different nutritional requirements can cause important differences in foraging, survival and performance of the sexes (Conradt 1998; Mysterud 2000;

Clutton-Brock et al. 1982). Therefore, it turns difficult for males and females to synchronize their activities and live in the same group, leading to sexual segregation (Ruckstuhl 1998).

Foraging activities (percentage of active deer feeding) may be related to sexual segregation (Bowyer & Kie 2004). However, sexual segregation may occur despite the differences in the diet as proposed by Conradt (1998) and Ruckstuhl (1998), which have shown that differences in foraging behaviour between males and females may lead to social segregation. Though, social segregation can happen independently of habitat segregation (Conradt 1998; Ruckstuhl & Neuhaus 2005). Regarding social segregation, one of the most accepted hypotheses is the activity budget hypothesis proposed simultaneously by Conradt (1998) and Ruckstuhl (1998).

Conradt (1998) proposed that sexual segregation can result from a lack of synchrony in activity between males and females, as well as Ruckstuhl (1998) that suggested that sexual segregation would be due to differences in activity patterns. The ABH assumes that differences in activity budgets between males and females can lead to social segregation (Ruckstuhl & Neuhaus 2005). One individual can compromise its own activity budget to synchronize with other group members. Since synchronization between males and females in their activities is energetically costly (Ruckstuhl & Neuhaus 2002), segregation seems to be the result of such cost. Heterogeneous groups, *i.e.*, groups with different age, size or sex classes will have a higher cost of activity synchronization. This synchronization in their activity might be lower when males and females are together, than when they are separated (Alves et al. 2013). Indeed, the best strategy will be to form same sex or size groups where the costs of synchronization are lower (Ruckstuhl & Neuhaus 2002). Differences in energetic requirements, activities and digestive abilities can lead to the absence of sexual synchronization, and then result into different forage behaviours and consequently, to segregation (Alves et al. 2013; Ruckstuhl & Neuhaus 2005). Different activity budgets occur between males and females with 20% or more of body size dimorphism (Ruckstuhl 1998; Ruckstuhl & Neuhaus 2000). Females will select habitats with high-quality food or spend more time foraging, while females with young might have different activity budgets depending on their energetic requirements (Alves et al. 2013).

The complexity and differences found between the described hypotheses lead to the assumption that there are many reasons and mechanisms involved in sexual segregation, and the results of some studies do not seem to provide support for FSH (Bonenfant et al. 2004; Alves et al. 2013). Our study emerged following the study of Alves et al. (2013), where it was not possible to assume that males use lower quality habitats than females, based only in differential use of habitat. A study based on forage ecology is needed to evaluate if there are differences in

the foraging selection between both sexes, and to understand if foraging behaviour is capable of fully explain the observed sexual segregation in this Mediterranean area.

1.2) Study species – Red deer *Cervus elaphus*

1.2.1) Species distribution

Cervus elaphus (Linnaeus 1758) is one of the most widely distributed mammals in the world (Salazar 2009). It is extensively distributed in Europe, but it is also present in Asia, Africa, and America (Fig.1). In Europe, many populations of cervids have increased over the last years (Lovari et al. 2008).

Fig 1: Distribution of red deer populations in Europe (adapted from Lovari et al. 2008).

In Portugal, although red deer populations were close to extinction, nowadays, are increasing in both abundance and geographical range (Salazar 2009). This increase is mainly due to habitat changes and reintroduction programs (Alves 2013).

Fig 2: Red deer distribution in Portugal (adapted from Salazar, 2009)

Red deer populations in Portugal (Fig.2) are present in various areas such as the Montesinho Natural Park, Lousã Mountain, International Tagus Natural Park , Moura, Barrancos, Tapada Real de Vila Viçosa and Tapada Nacional de Mafra (Salazar 2009).

1.2.2) Morphology, ecology and behaviour

Cervus elaphus is a wild animal and one of the largest ungulate and ruminant mammals of Europe. It belongs to the family Cervidae. Males and females have secondary sexual characteristics and sexual body-size dimorphism allowing individual recognition. The main observable differences between males and females, are 1) the presence of the antlers in males, 2) body shape and size, 3) coat colouration and 4) facial features (Peixoto 2014; Clutton-Brock et al. 1982). Females are significantly smaller than males, emphasizing the sexual dimorphism of this species (Alves et al. 2013). In Mediterranean regions, the red deer male is approximately 175 to 250 cm long and weighs 130 to 180 kg, while the female is 160 to 210 cm long and weighs 80 to 120 kg.

Red deer males have, in their skulls, natural bone formations called antlers, which are much prized by human hunters (Peixoto 2014). The antlers drop off every year after the breeding and winter seasons, around March, and develop again in the same year, which takes 4 months. Its growth is determined by hormones and has a high energetic cost (Peixoto 2014; Clutton-Brock et al. 1982). There may be a relationship between the age of the male and the number of antler tips, but this estimation is inaccurate because growth of antler is also influenced by the physiological state of the animal, food quality and genetic factors (Kruuk et al. 2002). On the other hand, age can also be easily assessed by teeth eruption patterns, determining the age of wild animals (Azorit et al. 2002). In terms of age, red deer individuals may be grouped in three age classes, “young” (less than 1 year), “sub-adult” (between 1-2 years in females and 1-3 years in males), and “adult” (higher than 2 years for females and 3 years for males) (Alves et al. 2013).

The red deer coat is usually thick, with seasonal variations in type and colour. These variations include brightness and tonality differences, wherein the hair appears reddish brown during the summer and dark brown during winter (Peixoto 2014). The offspring with up to 2 months old has dorsal white spots, which help camouflage in vegetation (Peixoto 2014).

Red deer is a polygamous species (Clutton-Brock et al. 1982) that presents a slightly gregarious behaviour, characterized by the sexual segregation between males and females outside the reproductive period (Alves 2013; Clutton-Brock et al. 1982). The reproductive cycle is divided into three main steps: 1) gestation from September to May, 2) lactation from June to September and 3) rut from September to October (Alves et al. 2013). The cycle is highly synchronized, with birth and reproduction occurring in very determine periods (Alves et al. 2013; Clutton-Brock et al. 1982).

Behavioural changes in the individual responses are observed along the life cycle. Before rut, adult males become intolerant with each other (Alves 2013), and began to move to the reproductive areas to define their territory. They become more interested in females and start to aggregate with them, which correspond to the formation of harems. Clutton-Brock et al. (1982) defined harem as the group of females defended by a male during the rut. The harem can be formed by at least one female and one male (Bonenfant et al. 2004).

Outside the rut season, red deer demonstrate a matriarchal society in which adult females live aggregate with sub-adults females and young, but segregated from adult and sub-adult males older than 3 years (Clutton-Brock et al. 1982).

Females in their second autumn can produce one, or very occasionally, two calves per year. Conception is followed by a gestation period of about 34 weeks, 240 to 262 days. The labour lasts between 30-120 minutes, and the offspring weight is about 15 kg (Clutton-Brock et al. 1982). After birth, the mother encourages the calf to follow her, usually moving away from their matrilineal group as a way of keeping the calves protected from possible predators. During this period, the mothers are intensively vigilant (Clutton-Brock et al. 1982). The lactation period is very important for both calves and mothers. Lactating females show reduced physical condition than the barren females, due to the maternal investment (gestation and lactation) which is energetically very costly, decreasing the mother's body-fat reserves (Clutton-Brock et al. 1982).

As well as females, males also have high energetic requirements, since they depend on their physical condition (accumulation of energy reserves as body fat) for the mating season and competition with other males, as well as antler growth (Clutton-Brock et al. 1982).

Males and females have different reproductive strategies. The success of females is dependent on the survival of their offspring, and thus they are more sedentary and passive, being considered as "conserver of energy" (Clutton-Brock et al. 1982). The reproductive success of males depends on physical condition in the rutting season, when they are more active in roaring, being thus classified as "expenders of energy" (Clutton-Brock et al. 1982; Ruckstuhl & Neuhaus 2005).

1.2.3) Feeding behaviour

In Mediterranean environments, red deer uses a high variety of habitats, whose reflects their resource requirements. This species shows a high plasticity which allows them to occupy a wide variety of habitats, including open areas like shrublands and grasslands (Alves 2013). However,

they can also occupy forestall habitats where they find protection and refugee (Lovari et al. 2008).

Red deer is considered as an intermediate feeder (Hofmann 1989), whereby they are able to be grazers, consuming mainly grasses, but also browsers, feeding on herbaceous and shrub foliage and trees. This variety is mainly dependent on food availability (Hofmann 1989). Red deer can adapt to seasonal changes in the food quantity and quality by changing the composition of their diet (Dumont et al. 2005). In summer, most of the herbaceous species become senescent, and red deer faces poor quality and lower availability of food, requiring them to browse on trees as a supplementary diet (Bugalho et al. 2001; Bugalho et al. 2005). In Mediterranean areas, the summer is a limiting period, that may lead to nutritional restrictions due to dry and hot weather (Alves 2013). When the first autumn rains arrive, the diversity of plants increases, increasing also its availability and biomass.

In Lousã Mountain (Portugal), red deer feed mainly on shrubs (Alves 2013), namely *Erica* sp., *Pterospartum tridentatum*, *Ulex* sp., *Rubus* sp.. However, they can also search for Gramineae species and arboreous species, like *Quercus* sp., *Castanea sativa* and *Pinus pinaster* (Alves 2013; Oliveira 2013). Depending on the time of the year, these animals will eat different structures of plants including roots, stems, leaves, flowers, fruits, sprouts and seeds, dependent upon the phenology and availability of plants.

Red deer males and females are sexually dimorphic, and due to that have different forage requirements as well as different energetic supplies. Those differences in body size may lead males and females to search for and use different resources (Ruckstuhl & Neuhaus 2005). To increase the foraging intake, males would typically choose grazing species and spend less time foraging than females (Ruckstuhl & Neuhaus 2005). To improve their performance during the rut season, males develop and improve their overall condition during the summer and spring months (Bonenfant et al. 2004).

Among the ungulates, partial migration including altitudinal movements is a common phenomenon (Qviller et al. 2013). Reproduction and birth seasons are well temporally established, so the nutritional requirements should change between seasons. Thus, red deer migrates to different altitudes throughout the year according to food quality and quantity (Clutton-Brock et al. 1982). In summer, they migrate to higher altitudes where they find better food sources (Bonenfant et al. 2004; Qviller et al. 2013), particularly in areas with snow melt. This is an adaptive behaviour through which animals are able to choose places as a way of minimizing energetic losses and maximize gains. Besides that, habitat use may be influenced by

physiological and behavioural responses to interspecific competition, predation risk, anthropogenic factors or environmental changes (Lovari et al. 2008).

1.2.4) Importance

Deer is one of the species with greater ecological and economic importance in Europe, being intensively studied over the last years (Clutton-Brock et al. 1982). However, there is a lack of adequate directives to its proper management in Mediterranean regions (Peixoto 2014). A better understanding of the selectivity of red deer in terms of food and habitat is crucial to manage populations and habitats (Dumont et al. 2005).

Red deer, like many other cervids, play an important role in plant dynamics through a selective intake of vegetation (Bugalho & Milne 2003). High densities of deer populations can cause serious problems in ecosystems, mainly in regeneration of young and plant growth especially as a consequence of browsing (Szemethy et al. 2003; Côté et al. 2004). In addition to the effect on vegetation, there are also influences on soil biota and nutrients, as well as in animal communities (Tolleson et al. 2005; Alves 2013). Deer faeces have a huge amount of organic matter, and largely consist of bacteria, undigested food, water, minerals and gastric secretions. Thus, being valuable for soil enrichment and microbial activity (Tolleson et al. 2005).

In terms of conservation, *Cervus elaphus,* is an essential element for maintaining stable populations of some protected species, namely some threatened carnivorous species like Iberian wolf (*Canis lupus signatus*) and Iberian linx (*Lynx pardinus*) (Cabral et al. 2005).

The economic income promoted by red deer is extremely valuable because of their value as game species due to their trophies (Peixoto 2014). They are also hunted for meat and medicinal products (Martinez et al. 2002; Milner et al. 2006).

1.2.5) Deer management

During the nineteenth and twentieth centuries, some activities such as over exploitation and the agriculture activities led to a decline of the red deer population in Portugal, both in terms of

number of individuals and distribution (Salazar 2009). After the 1960s, there was a re-establishment of the natural habitats of the species due to high levels of emigration, de-population of rural and agricultural areas, deer reintroduction programmes, creation of protected areas and regulation of the legal hunting activities (Salazar 2009).

Having been close to extinction, red deer populations in Portugal are now increasing in both abundance and geographical range (Salazar 2009). The Lousã Mountain was a target area of a reintroduction process that occurred between 1995 and 1999 where ninety-six animals were released (sixty-four females and thirty-two males) into the central area of the mountain (Salazar 2009; Alves 2013). Since the reintroduction programme, the population has increased in size and distribution, and the total area of red deer population is approximately 435km^2 and with a mean density estimated of 5.6 deer/km^2, between 2005 and 2009 (Alves et al. 2013).

As with other ungulates, the increase in both abundance and geographical range throughout Europe begins to conflict with human activities. These can only be minimized by proper deer management programmes. Indeed, high populations of deer may disturb agricultural lands, with consequent damage to crops and plants. The management of habitats is crucial to protect these species (Burbaite & Csányi 2009). Some essential aspects to consider in management are food availability, favourable areas for breeding and availability of refuges for protection of individuals against predators or adverse conditions (Peixoto 2014).

Historically, deer populations would have been controlled by natural predators. However, these natural predators became absent and deer populations became uncontrolled. Wild dogs became a non-natural predator preying mainly on young, sub-adults and adult female deer. In order to control deer population, hunting activities also increased. In the Lousã Mountain, hunting started in 2006/2007. Nowadays, this region includes twelve hunting areas, seven of which include the hunting of Red deer being the process called “montaria”. The hunting season, occurs every year from October to February, outside the central part of the Lousã mountain (Alves et al. 2013). Profit from deer hunting can only be achieved with a proper management of the populations.

1.3) Study area

1.3.1) Location, climate and topography

The study area is the Lousã Mountain which is located in the central region of Portugal (40°3'N, 8°15'W) with an approximate area of 170km^2 (Alves et al. 2013). The mountain altitude range from 100 to 1205 m above sea level with deep valleys and pronounced hilltops (Alves 2013).

The mountain is characterized by a Mediterranean climate with hot and dry summers and rainy winters (Archibold 1995). Temperature and precipitation patterns vary due to the mountainous topography as a consequence of slope, altitude and landscape which create climatic differences (Alves 2013). The annual mean temperature is 12°C, varying between -4.1°C and 35.9°C. The mountain has a dense hydrologic network belonging to the drainage basins of the Mondego and Tejo rivers.

Lousã mountain has a vast road network with more than 500 km, with low traffic levels. The area is more disturbed in the beginning and the end of the day, which are the periods with higher traffic.

1.3.2) Land cover and flora

Concerning land cover, the Lousã mountain is characterized by mixed habitats composed by coniferous and broadleaf tree plantations and also large areas of shrubland. The coniferous forests consist on species of pine trees (*Pinus pinaster*, *Pinus sylvestris* and *Pinus nigra*), *Pseudotsuga menziesii* and also Mexican cypress (*Cupressus lusitanica*). The broadleaf trees are less common and constituted by *Quercus* sp., *Castanea sativa, Prunus lusitanica* and *Ilex aquifolium.* In the shrubland areas we can find *Erica* spp., *Calluna vulgaris, Ulex minor, Rubus ulmifolius* and *Pterospartum tridentatum* as the dominant and most frequent species. Other plant species that can also find *Genista triacanthos, Halimium umbellatum, Lavandula stoechas* and *Lithospermum diffusum.* Riparian zones are also present near the water courses with *Alnus glutinosa*, *Betula* spp. and *Salix* spp..

At the lowest elevations, the most frequent species are plantations of eucalyptus trees (*Eucalyptus globulus*) alone or mixed with *Pinus pinaster.* These plantations are dominant outside the mountainous region. During recent years, *Acacia melanoxylon* e *Acacia dealbata* (exotic species) abundance has increased (Alves 2013).

Gramineae species are also very important in red deer diet and they are present in large quantities. In Lousã mountain we also have forb species, that although not abundant are very diverse (Alves 2013). Some examples of those are *Anarrhinum bellidifolium, Carduus tenuiflorus, Crepis vesicaria, Digitalis purpurea, Juncus effusus, Lepidophorum repandum, Lepidophorum officinale, Tuberaria lignosa* and *Genista triacanthos.*

1.4) Aims

The main goal of this study is to evaluate if the differences in the diet of sexes are enough to explain the sexual segregation. To achieve the goal, it is important to evaluate the composition, diversity and quality of the diet of males and females of red deer in the Lousã Mountain.
More specifically, it is intended to study which factors lead males and females to choose one habitat/space to feed rather than another and why they are segregated all year except the rut season. It will be crucial to understand if the different nutritional requirements between the sexes are behind the segregation among males and females. There are many hypotheses attempting to explain this phenomenon, however, the focus of this work will be on the Forage Selection hypothesis, to address how far this hypothesis can explain the sexual segregation.

1.5) Thesis framework

This thesis is divided into five chapters. The first, and the present chapter present a general introduction on the subject sexual segregation. An introduction to sexual segregation as well as to the different hypotheses that might explain it are tackled. Red deer in terms of their population and distribution, morphology, ecology, behaviour, foraging ecology, importance and deer management are also addressed. In addition, this chapter includes a description of the study area, the Lousã Mountain (Portugal).

Chapter II is a methodological chapter which includes the analysis of the required sampling effort when using the microhistological technique. The main goal of this chapter is to determine which would be the optimum number of fragments to study the forage composition of red deer.

Chapter III “Can feeding behaviour of males and females be the reason behind sexual segregation in red deer?”, concerns on feeding ecology of red deer and attempts to approach the main goal. Diet in terms of composition, diversity and quality is assessed. In this chapter, a relation between the sexual segregation and the forage ecology, including different nutritional requirements is found. A discussion on the ability of the Forage selection hypotheses to explain sexual segregation is presented.

Chapter IV includes the main conclusions of the work concerning red deer feeding ecology and also sampling effort, as well as guidelines for future works regarding sexual segregation. In the last chapter, V, are comprised all the references.

Chapter II- Methodological notes – Sampling effort

2.1) Introduction

Composition, characteristics and habitat of feeding of mammals are very interesting for biologists and ecologists (Mátrai et al. 1998). Analysis of the botanical composition of the diet of herbivores can be done by variety of methods (Holechek et al. 1982; Holechek 1982; Sanders et al. 1980). Microhistological technique was initially developed by Baumgartner & Martin (1939) and latter refined by Sparks & Malechek (1968), and became the most commonly used indirect method for determining herbivore diet (Holechek & Vavra 1981; Holechek et al. 1982). Microscopic examination of faecal material is inexpensive, requires low equipment, after training is accurate, and produces good overall results, allowing the identification of each plant species consumed (Holechek 1982; Sanders et al. 1980). Also, it does not interfere with the normal habitat of the animals, and can be used to compare diets of more than one individual (Holechek & Vavra 1981; Holechek et al. 1982; Maia et al. 2003). This technique is based on the identification of plant fragments, by comparison with a reference collection of epidermis, like the one presented in Annex 1 (Sanders et al. 1980; Holechek et al. 1982; Szemethy et al. 2003). Although the usefulness of the reference collection in identifying the plant fragments, it is a disadvantage of this technique because it is a very time consuming procedure (Ahmed et al. 2015). Therefore, using a dichotomous key (see Annex 2) based on microhistological features of each individual structure of plant species is a good strategy to minimize the time spent in identifications (Carrière 2002; Ahmed et al. 2015). Other disadvantage of the microhistological technique is the time required to identify the plant fragments (Holechek & Vavra 1981; Holechek et al. 1982; Carrière 2002; Maia et al. 2003; Ahmed et al. 2015; Dove et al. 1995) that may be minimized by using the appropriated number of fragments and samples.

In order to overcome this shortcomings, assess a minimum sampling effort, meaning the minimum number of fragments needed to assess a given precision, determined based on the objectives of each study, must be determined in order to save time and money. Indeed, sample size could affect the estimate of several sampling phases of microhistological technique such as 1) animals, 2) pellets and 3) epidermis fragments (Katona & Altbäcker 2002).

The required minimum sample size increases with the increase of diversity of diet and with the heterogeneity of the individuals (Kovács & Török 1997). It could also vary from study to study depending on size and characteristics of the study area, as well as with study species (Anthony & Smith 1974). Determination of the sample size required to effective assess diet composition analyses is difficult and there is no agreement about the minimum sample size needed (Katona & Altbäcker 2002). Indeed, different number of fragments needed are proposed by different

authors. Katona & Altbäcker (2002) suggested the analyses of 100 fragments as an optimum for estimating forage classes. On the other hand, Maia et al. (2003) suggested the analysis of 4 faecal pellets, using 100 plant fragments.

The main goal of this methodological chapter is to determine the optimal number of plant fragments needed to access the red deer diet by the microhistological technique. We hypothesize that sampling effort is positively related to species richness.

2.2) Material and methods

A total of 141 faecal samples and 200 fragments per each sample were analysed (for further sampling details see the procedures explained in detail in the "Materials and methods" of chapter III). The faecal samples are considered in this analyses as replicates, and in each sample, the 200 fragments were divided into groups of 10 (one microscopic slide).

The data analysis was divided into the following phases: 1) species accumulation curves, 2) rarefaction curves, 3) predictive models and 4) determination of sampling effort. These procedures were done using the function "Speccaccum" in the "Vegan" package in "R" software (Gotelli & Colwell 2011; Kindt et al. 2006).

There are a variety of methods to calculate the values of expected richness (Gotelli & Colwell 2011). Based on model fitting, a negative exponential model was chosen, given the capacity of reaching an asymptote and low number of parameters (Shiu & Lee 2003; Gotelli & Colwell 2011; Moreno & Halffter 2001). The negative exponential model is represented by:

$$S(t) = \frac{a}{b}[1 - \exp(-bt)]$$

where $S(t)$ is the number of species at t accumulated fragments, a and b parameters regulating the shape of the curve and a/b the asymptote of a given sample, *i.e.* maximum predicted number of species (adapted from Moreno & Halffter 2001; Shiu & Lee 2003). As soon as this asymptote is reached, the species accumulation curve is flat and the analyses of more fragments will not profit any additional species (Gotelli & Colwell 2011). Indeed, it means that sample size is large enough and no more fragments are needed (Kovács & Török 1997).

To achieve the sampling effort, *i.e.* the number of fragments require for a given percentage of total richness, was applied a variation of the negative exponential model, represented by:

$$t_q = -\frac{1}{b}\ln(1-q)$$

where q is a value between 0 and 1 (a proportion of the asymptote) and t_q the number of fragments needed to sample a q fraction of the total richness (adapted from Moreno & Halffter (2001); Shiu & Lee (2003)). We calculated sampling effort needed for 60%, 70%, 80%, 90%, 95% and 99% of the species richness.

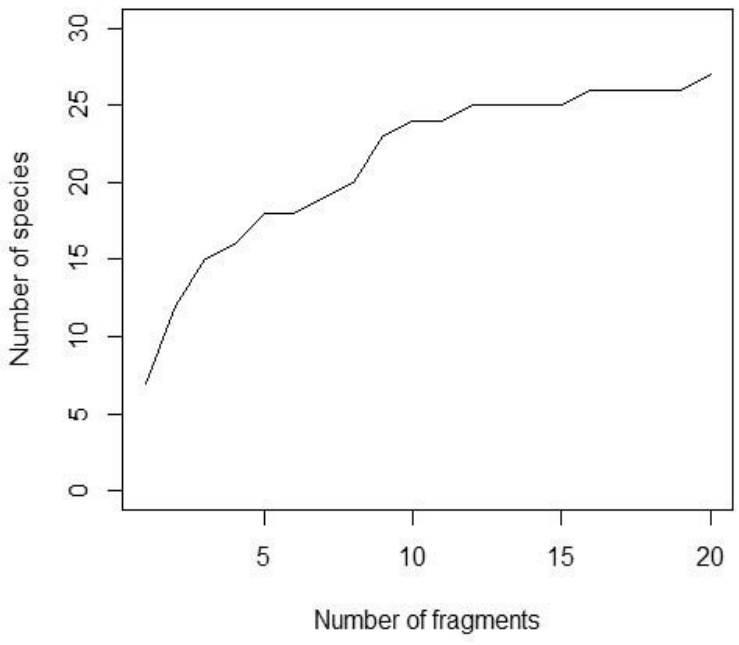
The measurements were performed in R. The results are presented as mean ± standard error, unless otherwise stated.

2.3) Results

2.3.1) Accumulation curves

Accumulation curves represents the number of species recorded by the number of fragments analysed (Gotelli & Colwell 2011; Kovács & Török 1997; Moreno & Halffter 2001).

a)



b)

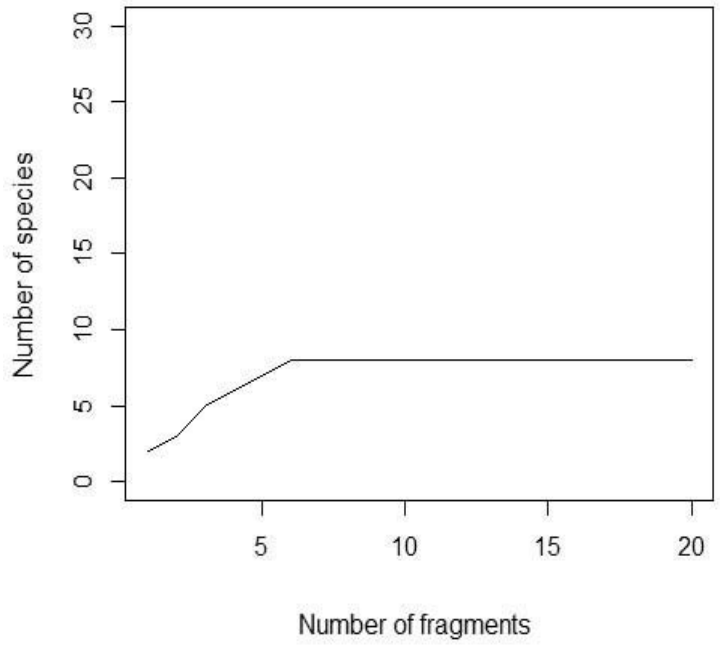


Fig 3: Example of two accumulation curves of two different samples. a) Accumulation curve for the sample with the highest number of different species recorded (27 species). b) Accumulation curve for the sample with the lowest number of different species recorded (8 species).

From our results it is possible to assume that the number of species recorded in each sample is very heterogeneous (individual) (Fig. 3). As we can observe from the accumulation curves, the number of species can vary from 8 up to 27 species (Fig. 3, b) and a) respectively).

2.3.2) Rarefaction curves

Accumulation diversity of species was calculated from blocks of 10 fragments, randomly analysing the variability inter and intra samples. To measure this variability, rarefaction curves were performed (Fig.4).

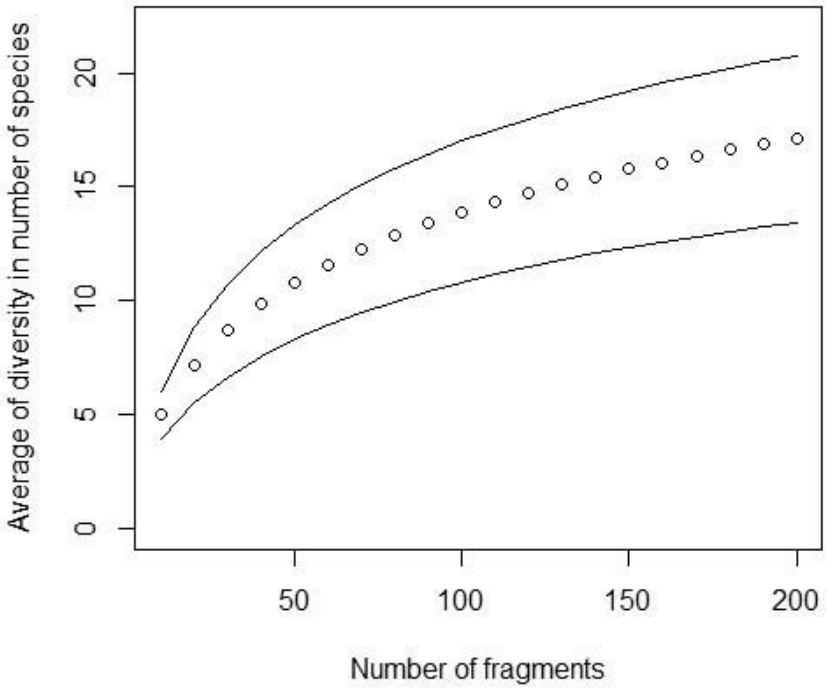


Fig 4: Sample based rarefaction curve of the mean of all 141 samples. Empty circles represent the mean and solid lines represent mean ± the standard deviation.

In this rarefaction curve, we can see the average of accumulated diversity in the observed number of species within 200 fragments for all 141 samples. Our results showed that with the total number of 200 analysed fragments per sample it was detectable 17.11 ± 3.76 species for all the samples. It is expected that the number of species richness increases with the increase of fragments analysed. However, from about 160 fragments, the species richness recorded begins to stabilize.

3.3.2) Sampling effort

The rarefaction curves give us results based on our data, the predicted species richness and the sampling effort may be calculated. The total number of species sampled, the asymptote, the % of asymptote and the R^2, fitted by the Negative exponential model, were calculated.

The parameters a and b needed for this model were estimated and the asymptote was then calculated (a/b). The value of the predictive asymptote was 16.54 ± 3.64. Indeed, the predictive asymptotic values were similar to the total number of species observed $(17.11 \pm$

3.67). The results of R^2 are considerably high (0.95), consequently this model is adequate for our data.

Our results predicted, on average, that 140 (140.25 ± 37.50) fragments would be sufficient to represent 95% of the species richness of the samples and 215 (215.62 ± 57.67) fragments would be necessary to represent 99% of the species richness of the samples (Fig.5).

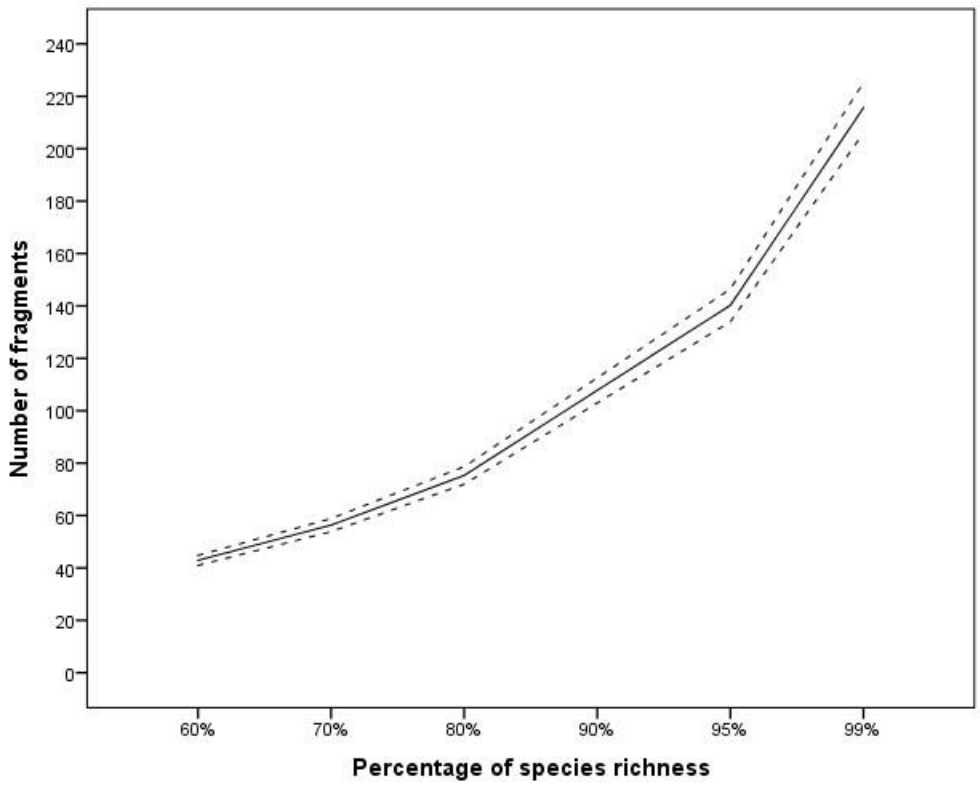


Fig 5: Average of sampling effort needed to reach 60%, 70%, 80%, 90%, 95% and 99% of the total predictive richness. Dashed lines represent the confidence intervals.

The sampling effort was also assessed for the different seasons (Fig.6). Our results demonstrate that to reach 99% of the total predictive richness, more fragments would be needed in the winter season followed by the rut and autumn seasons. On the other hand, with the 200 analysed fragments in the spring season, it is possible to achieve the 99% of the total predictive richness. In all seasons, for at least 95% of the species richness, the 200 analysed fragments were enough.

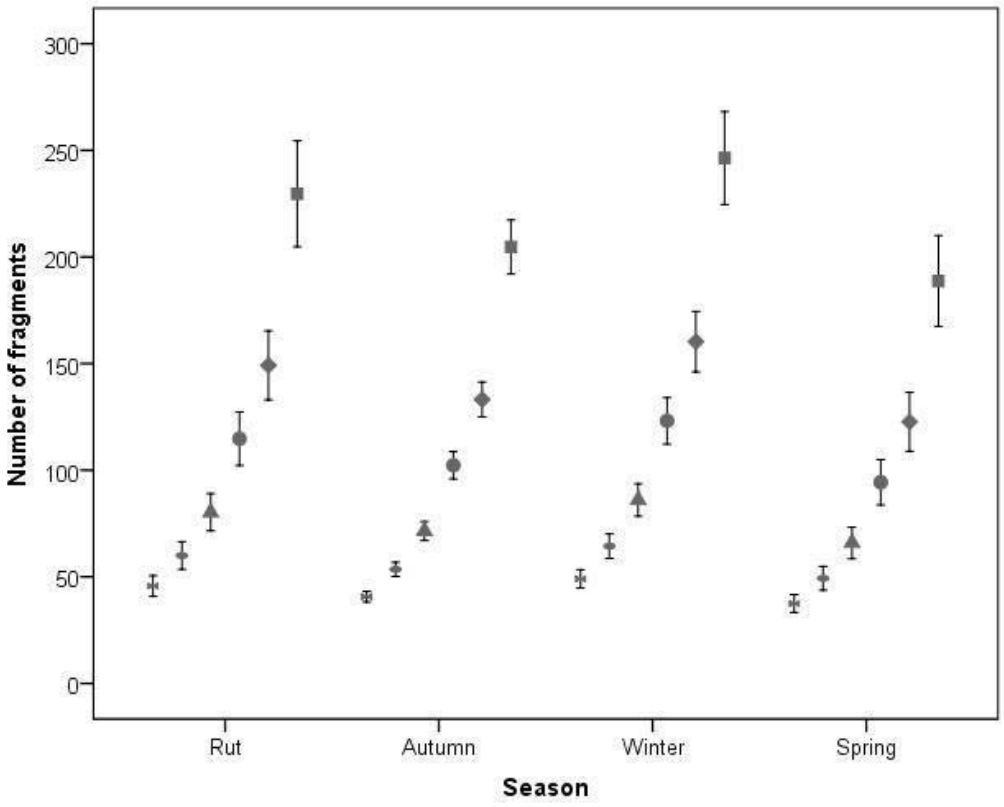


Fig 6: Average of sampling effort needed to estimate 60% (horizontal hourglass), 70% (rectangles), 80% (triangles), 90% (circles), 95% (diamonds) and 99% (squares) of the total predictive richness per each season. Solid lines represent the confidence interval.

2.3.4) Time effort

In each microscopic slide 10 fragments were photographed. A total of 20 microscopic slides per sample were made, making the total of 200 fragments analysed. Time consumed for each microscopic slide was calculated. There are a total of 31 samples represented in the figure 7 that were randomly chosen, following the Central Limit Theorem (Gonçalves et al. 2000). From these 31 samples, 14 are females, 11 males and 6 calves.

Our results establish, as expected, that the time needed to identify the first microscopic slides is higher compared to the last slides. In the beginning of the identification, when more different species appear, more time is spent consulting the reference collection and the dichotomous key to identify the fragments that appeared in the faeces. As more fragments are identified, the number of new species began to decrease and less time is spent (Fig. 7).

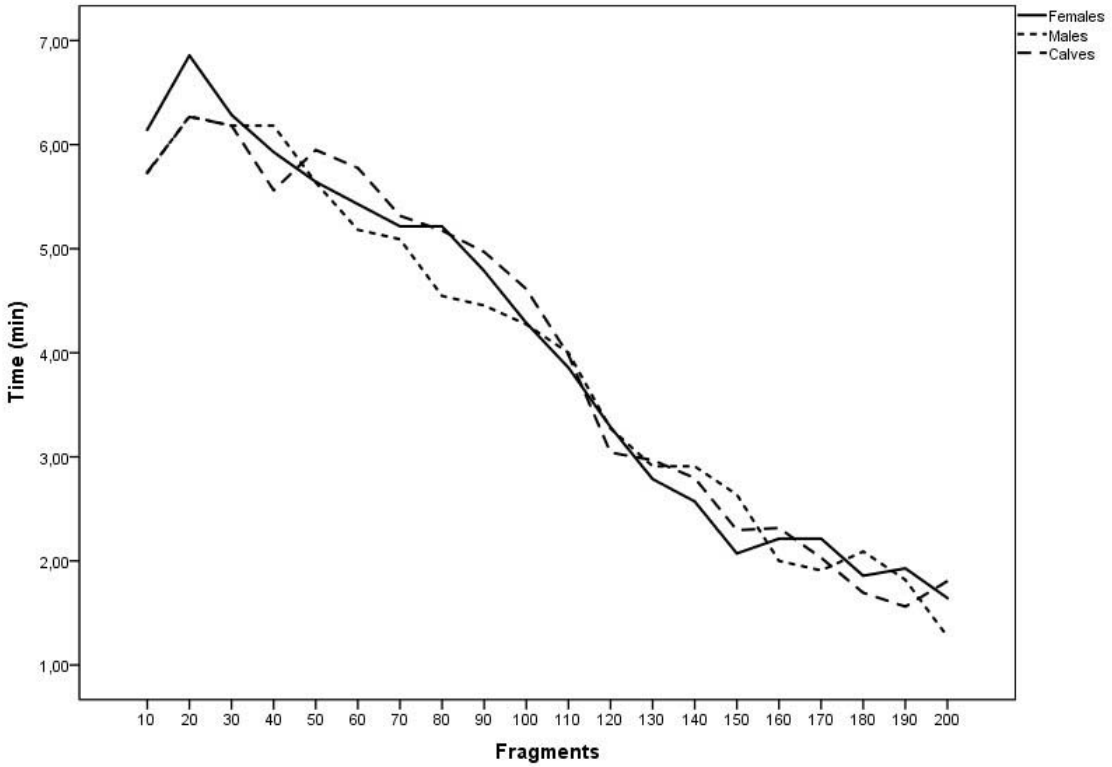


Fig 7: Representation of time spent identifying each microscopic slide from 31 random samples of females, males and calves.

2.4) Discussion

From the 141 samples analysed is possible to conclude that the number of plant species consumed by red deer is quite heterogeneous. The values of standard deviation seen in the rarefaction curve can be explained by this heterogeneity in the number of species recorded between the samples. It is known that higher heterogeneity of samples will require a larger number, in this case of fragments, to achieve the same relative number of precision in species richness (Gotelli & Colwell 2011). Furthermore, according to Flather (1996), fewer species would represent that a higher proportion of the total plant species would be found in less time. Indeed, some authors, in studies with other herbivores (Chapuis 1980) have suggested that the pressure on the vegetation by a given herbivore is the reason for these differences, rather than the actual food choice of the individuals.

We applied one predictive model that fitted this variability and at the same time captured the asymptotic behaviour showed in the rarefaction curve. There has been much discussion about the right model that should be applied to each data set (Soberón & Llorente 1993; Ugland et al.

2003; Gotelli & Colwell 2011). Some authors have suggested the size of the study area as the criteria for choosing the model. For example, Palmer (2012) analysed plant species in a forest and concluded that "an ecologist interested in comparing species richness can chose any estimator except the log-log model". However, Kerbs (2001) analysed species richness and concluded that "the exponential model is only appropriate for small sampling areas, the logistic model is the best for large-scale and power model for intermediate sampling areas". Nevertheless, there is no correct model that should be applied, as it depends on the fit of the models to each dataset. Two or more functions may be fitted to a specific dataset equally well, but can differ drastically in the estimation of asymptotic richness (Soberón & Llorente 1993; Chao et al. 2005). In our analysis, it was applied the negative exponential model, that was chosen among the others for the reasons previously exposed in the section "Materials and methods" of this chapter.

Microscopic examination of faecal material has become the most commonly used method for determining herbivore diet (Sanders et al. 1980; Holechek 1982). However, it has the limitation of time needed to analyse and to identify all the fragments. Furthermore, many authors suggest different number of fragments needed (Katona & Altbäcker 2002; Maia et al. 2003).

Katona & Altbäcker (2002) suggested the collection of 10 independent droppings, 1 pellet/individual and the analyses of 100 fragments as an optimum for estimating forage classes. On the other hand, Maia et al. (2003) performed various analyses to access optimal sampling schemes for the estimation of red deer diet and suggested that the analysis of 4 faecal pellets using 100 plant fragments would offer a good compromise between precision and cost. Also, their results showed that using a total of 6 pellets groups, the same precision could be obtained analysing only 30 plant fragments.

The composition and level of heterogeneity, size and characteristics of the study area as well as study species (Anthony & Smith 1974) and available types of plants could be the factors behind those differences between the numbers of fragments suggested. The study area of Katona & Altbäcker (2002) is in Hungary, a protected area composed by shrubland vegetation. In the case of Maia et al. (2003), the study area is in Vila Viçosa (south Portugal), a region characterized by large and flat landscapes covered mostly by holm oak, cork oak and olives. In our case, the study area is composed of a large variety of species ranging from arboreous to herbaceous species with a high variety of shrubs. Since the required minimum sample size increases with the diet diversity (Kovács & Török 1997), the sampling effort for study red deer diet in our study area may be higher due to plant diversity available. Indeed, the higher number of fragments

necessary to reach 95% of the total predictive richness of species in winter and autumn could be related with plant availability.

Analysing this amount of fragments is time-consuming (Holechek 1982) and defining a threshold between cost – precision is necessary. In fact, a total of 5h05min was needed per each faecal sample. Such time was divided in four main phases: 1) the preparation of the sample in the electric blender consumed a mean of 5min per sample; 2) the examination and photographing of all 200 plant fragments in the microscope took approximately 3h; 3) the identification of all plant fragments present lasted 1h30min; and 4) the introduction of all data in the excel matrix for further analysis was carried out for about 30min for each sample. The diet was assessed in 141 samples. Multiplying the time consumed in each sample by the total samples, a total of 722.15h were spent analysing samples. Considering that a day of work had about 9h30min and assuming that each month has 22 days of work, it took approximately 3 months and 9 days to analyse all the samples. Besides these 3 months, there was an initial training period of approximately 1 month.

To overcome this problem, we measured the sampling effort to reach 80%, 90%, 95% and 99% of the total richness consumed by red deer in the Lousã Mountain. Our results show that our sampling scheme was adequate, and the 200 fragments analysed is a proper effort for the study aims, because it provides a good representation of the plant species consumed. Thus, to analyse 200 fragments seem to be a good compromise between precision and cost, mainly because an effort of 200 fragments does not imply the double of time that 100 fragments would require.

However, is important to understand that the optimum number of sample size will depend on the aims of a particular study. If the objective is to study the diet of an herbivorous species at a general level, lower number of fragments can be analysed. Nevertheless, if the aim is to analyse the diet composition on a species level and diversity, a higher number of fragments would be necessary.

Since time and costs are the major limitation in research programs, assess the sampling effort schemes could be a very useful tool to optimise the analyses, defining a good compromise between precision and cost.

Chapter III- Can feeding behaviour of males and females be the reason behind sexual segregation in red deer?

3.1) Introduction

Forage selection hypothesis (FSH) described by Bowyer (1984), predicts that sexual body-size dimorphism causes differences in sex-specific nutritional requirements that are related to food selection and subsequently led to habitat segregation (Ruckstuhl & Neuhaus 2000; Barboza & Bowyer 2000). Gut capacity increases proportionately with body mass, whereas metabolic requirements decrease with the increase of body mass (Demment & Van Soest 1985). Males might prefer habitats with lower quality forage but with higher biomass (more ubiquitous and fibrous plants) because they are good at digesting fibres due to a larger rumen and slower passage rate of food. On the other hand, females might choose habitats with lower quantity but higher quality of forage (high nitrogen and low fibre levels) because they are less efficient at digesting owing to a small stomach size and lower gut capacity (Bowyer 2004).

Assuming that this is truth, the females would need to compensate this inferiority of the digestive system by either two ways, 1) selecting higher-quality forage than that ingested by males, or 2) increasing foraging efficiency (Bailey et al. 1996). Moreover, transfer of nutrients and energy costs of lactating females led them to select food sources with high levels of sodium, calcium or nitrogen (Clutton-Brock et al. 1982). Moreover, sexually active females have higher energetic requirements due to gestation and lactation than males or barren females. They should, then, opt by higher-quality food, which may result in their segregation from non-sexually active females and males, due to the selection of different plant species or habitats with different availability of nutrients (Clutton-Brock et al. 1982).

Different assumptions need to be made for assuming this hypothesis as true: 1) segregation by space, 2) difference in plant selection in the same habitat or 3) low overlap of the habitat (Ruckstuhl & Neuhaus 2000). Moreover, differences in foraging behaviour of the sexes should increase with the increase of sexual body-size dimorphism (Ruckstuhl & Neuhaus 2000).

Nevertheless, forage selection hypothesis is controversial (Ruckstuhl & Neuhaus 2000). Over the last decades, several studies regarding sexual segregation in ungulates have been made, some corroborated the assumptions of FSH, in which females will select higher quality food habitats than males (Mysterud 2000; Pérez-Barbería et al. 1997), but others verified the opposite, rejecting FSH (Miquelle et al. 1992; Clutton-Brock et al. 1982; Main & Coblentz 1996; Ruckstuhl 1998; Bonenfant et al. 2004). Several authors are questioning the capacity of FSH to explaining all the patterns of sexual segregation observed in ungulates by itself, indicating that there are various reasons and mechanisms involved in this phenomenon, including social factors and

other habitat requirements, other than feeding behaviour (Bonenfant et al. 2004; Alves et al. 2013).

Red deer are considered as an intermediate feeder (Hofmann 1989), whereby they are able to be a grazers, consuming manly grasses and sedges, but also to be a browsers feeding on forbs and shrubs foliage and trees, depending on food availability (Bugalho et al. 2001; Gebert & Verheyden-Tixier 2001; Bugalho & Milne 2003; Szemethy et al. 2003; Ruckstuhl & Neuhaus 2005; Dumont et al. 2005).

Red deer males and females have differences in energetic requirements caused by different body sizes, that may lead males and females to seek and use different resources (Mysterud et al. 2004; Ruckstuhl & Neuhaus 2005). In Mediterranean environments, the lack of quality food during early summer may affect the reproductive success of males during stems production and recover the fat reserves lost during the rut season, as well as females, especially if they are lactating (Bugalho & Milne 2003; Szemethy et al. 2003; Putman & Staines 2004).

Morphology and chemical composition of plants are also reasons for different diet compositions and for the consumption of a wide range of plant species (Chevallier-Redor et al. 2001). Differences in availability of food can promote movements and alteration of habitat use (Szemethy et al. 2003; Ceacero et al. 2012). Red deer may adapt to seasonal changes in terms of both quantity and quality of food, and environmental conditions by changing the composition of their diet (Hofmann 1989; Bugalho et al. 2001; Bugalho & Milne 2003; Dumont et al. 2005). Depending on whether the resources are consumed in similar proportions to those that occur in the habitats or not, its presence in the diet of red deer could be a result of a selective process or just a reflection of their abundance (Bugalho & Milne 2003).

Red deer migrates throughout the year to different altitudinal levels, as a response to changes in the quality and quantity of food (Clutton-Brock et al. 1982). This species shows a high plasticity, which allows them to occupy a wide variety of habitats, including open areas like shrublands and grasslands (Szemethy et al. 2003; Alves 2013). In early summer, red deer move to higher altitudes where they find better food supplies, after snow melts (Bonenfant et al. 2004). In this season, most of the herbaceous species become senescent, and red deer faces a period of poor quality and lower availability of food, requiring them to browse other vegetation resources (Bugalho et al. 2001; Bugalho & Milne 2003). Bugalho et al. (2001), predicted that the individuals with a larger body size (males) will have higher physical ability to reach the trees, and to profit from browsing on arboreous species in more limiting seasons, whilst, a smaller body

size may be beneficial when grazing. This is an adaptive behaviour whereby animals are able to choose the places as a way of minimizing energetic loses and maximise gains.

The selection of food items by the animals is closely linked to the phenology of plants but also to their nutritional and energetic requirements (Chevallier-Redor et al. 2001). Indeed, the nutrient status of herbivores are related with the nutritive value of plants, the intake of the animal and the botanical composition of the diet consumed (Dove et al. 1995). Photosynthetic pigments, meaning chlorophylls and carotenoids, are used to quantify photosynthetic capacity of the plants present on faeces and then assess food quality (Christianson & Creel 2015).

The main aim of this study was to evaluate the feeding behaviour of males and females of red deer in the Lousã Mountain, and to infer if there are enough differences in the diet composition, diversity and quality between the sexes capable of explaining sexual segregation. Based on the microhistologic technique for the analysis of faecal samples, we expect to identify the differences in the diet composition and diversity of males and females, and to analyse if those differences are related with the patterns of sexual segregation observed for the studied population (Alves et al., 2013). If the feeding behaviour patterns show a relation with sexual segregation patterns, this study will provide support to FSH. Furthermore, using analysis of chlorophyll and carotenes, we expect to verify the existence of differences in the quality of the plants consumed by both sexes, which may also indicate a support to FSH.

3.2) Materials and methods

3.2.1) Study area and collection of plants and faeces

The study area of this work is the Lousã mountain which is located in the central region of Portugal (40°3'N, 8°15'W), with an approximate area of 170km^2 (Alves et al. 2013). The study area is characterised by a Mediterranean climate (Archibold 1995).

The plant species were collected in the Lousã Mountain between 2014 and 2016, and the focus was to collect the maximum number of species that may be part of the red deer diet. The different plants were cut and placed into different plastics bags, to prevent any mix and to be easier to transport to the laboratory. The woodiest plants were collected with the help of a sharp object.

The collected plant species were divided into three groups: arboreous species, shrub species and herbaceous species. The herbaceous species were differentiated into dicotyledons and monocotyledons. The arboreous species are woody plants usually greater than five meters' height. The shrub species are woody plants with less than five meters' height without a main stem and with branching off from the base. Herbaceous species are usually small plants whose stem has little or no lignification.

The faecal samples were collected between 2014 and 2016. The collection of faeces was carried out at different sampling points in the Lousã mountain (Fig.8). The faecal samples were collected through direct observation of the animals defecating (wherein a total of 37 samples were collected, being 24 from spring, 10 from autumn and 3 from rut) and from hunted animals in "montarias" (hunting events, typical of the Iberian Peninsula).

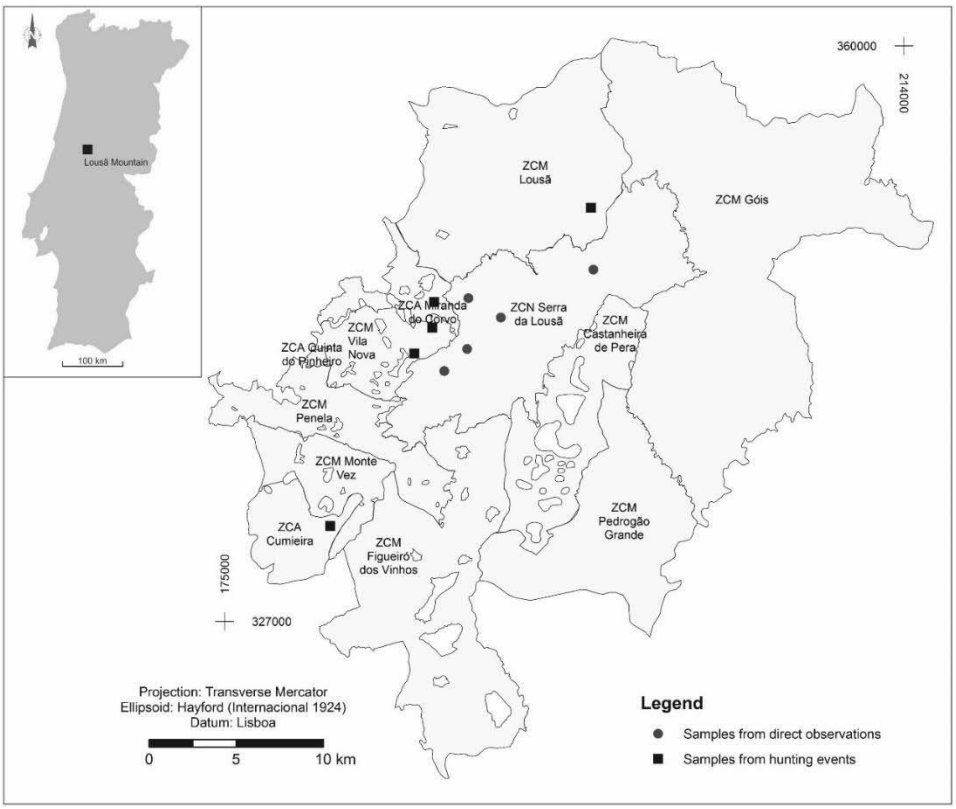


Fig 8: Map of the study area and sampling points.

Direct observations were made to identify the sex and age of each individual to which the samples belong. When one individual was observed, the register of its characteristics in terms of sex and age classes was made, and the observation of the animals was made with the help of binoculars and telescope until defecation. After the defecation of the individual, geographic

reference points, distance and bearing relatively to the observation point were recorded to enable the collection of the faeces. After being identified by sex and age, the faeces samples were placed into plastic bottles, properly identified and frozen in the laboratory at -20°C.

The faecal samples obtained from red deer hunted in "montarias" were collected in four different hunting areas: ZCM of Lousã, ZCM of Vila Nova and ZCM of Miranda do Corvo and ZCM of Cumieira, located in the Lousã mountain area (Fig. 8). A total of 104 faecal samples were collected between October and February in 6 hunting events (36 samples from winter, 34 from rut and 34 from autumn). The faecal samples were collected *in-situ* directly from each individual. This method allowed us to easily identify the sex and age of each individual. After being identified by sex and age, the faecal samples were placed into plastic bottles, properly identified and frozen in the laboratory at -20°C.

A total of 141 faeces samples were collected from different individuals, being 51 samples from males, 64 from females and 26 from calves.

3.2.2) Reference collection of epidermis and dichotomous key

For applying the microhistological technique in the analyses of faeces, a reference collection and dichotomous key were done (for more details see Annex 1 and 2, respectively).

The plants collected were identified to the species and all the different plant structures (stems, leaves, flowers and sprouts) were cut and placed separately in properly labelled bottles, in a solution of sodium hypochlorite. The structures remained in the solution for between 18 and 48 hours, depending on the physiology of the species (Maia et al. 2003). This procedure is done to make the observation and identification of all epidermal characteristics easier and to facilitate the detachment of the epidermis of the adjacent tissue. The process is complete when almost all the fragments are white. After this period, the solution of sodium hypochlorite was removed, and the plant fragments were washed. All the plant material was, then, placed into individual bottles with water for storage, the sodium hypochlorite that remains in the tissue acts as a preservative.

To prepare the epidermis, and before mount them in the slide, the mechanical detachment of the plant epidermis was performed. The pieces of the structures were placed in a Petri dish with water and the epidermises were separated from all the adjacent tissues (*e.g.* parenchyma). This was performed with tweezers and scalpel with the help of a magnifying glass (Bauer et al. 2005).

The epidermis tissue was mounted as a pre-definitive microscopic slide with glycerine (which helps whiten the epidermis), covered with a cover slip and sealed with varnish (Maia et al. 2003; Butet 1985). All pre-definitive slides were observed and photographed on bright-field (BF) and phase contrast (CF) microscope with a standard magnification of 200x and 400x with the programme “Leica application suite V4.6”.

The use of the reference collection and the dichotomous key allows the identification of the fragments through the use of individual characteristics and morphological features. The epidermis can vary greatly in form, size and organization between the different structures of the plant, namely in the organization and size of epidermal cells, the inclusion of crystals, sharpness of leaf marge and venation (Holechek & Gross 1982; Adulyanukosol & Poovachiranon 2003; Bauer et al. 2005; Barclay et al. 2007; Ahmed et al. 2015). There are also specialized epidermal cells such as stomata and trichomes that vary in size, shape or presence and position (Barclay et al. 2007; Toral et al. 2010). There are six different types of stomata: anomocytic, anisocytic, diacytic, paracytic, tetracytic and ciclocytic (Cotthem 1970). Different types of trichomes were also considered to construct the dichotomous key namely tector, secretory, starry, stinging, scaly and glandular trichomes (Zapater et al. 2009). Those features are essential for the identification of fragments.

3.2.3) Diet composition

For the analyses of the diet composition the microhistological technique was applied following the methods described by Sparks and Malechek (1968). Individual faecal samples were defrosted and five randomly individual pellets were mixed in 400ml of water in an electric blender for 10 seconds pulses, during 30 seconds (Sanders et al. 1980; Holechek & Vavra 1981; Szemethy et al. 2003; Maia et al. 2003). Automatic maceration is more advantageous since it makes the fragments more homogeneous in terms of size and distribution, and turn the separation of the epidermis from the adjacent tissues easier (Maia et al. 2003). The mixture was washed through a 0.075 mm sieve to remove any dirt and fragments too small (Sparks & Malechek 1968). A minimum dimension of 1 mm^2 of the epidermis should be guarantee to be identifiable (Maia et al. 2003). The material was moved into a Petri dish with sodium hypochlorite solution which helps whiten the material (Maia et al. 2003; Butet 1985). To get random microscopic slides, the Petri dish was placed on top of a properly numbered matrix, and a random algorithm to choose the numbers of the matrix to be sampled was used.

From the five pellets, twenty microscopic slides were prepared and ten fragments identified in each slide, in a total of 200 plant fragments for each individual. The identification of the fragments was made following systematic and alternate transects across the slide to avoid the duplication of fragments (Maia et al. 2003). All the fragments were examined and photographed at a magnification of 100x and 400x (the same magnification of the photographed epidermises of the reference collection). The identification of epidermal fragments present in the faeces was done by comparison with the photographs, with individual description made for each epidermal plant species and through the use of the dichotomous key.

Only fragments with four or more cells were used. Therefore, the unidentified fragments were fragments that do not have enough distinctive characteristics or that the species they belong to are not present in the reference collection of the epidermis (Bauer et al. 2005).

3.2.4) Diet quality

Nutritional analysis was access by the analyses of the concentration of carotenoids and chlorophylls using absorption spectrophotometry techniques as described in Christianson & Creel (2009). First, we started by drying the samples of faeces in an evaporator for 24h. Each 0.2g subsample of faeces were boiled in 95% ethanol for 15min. The pigment supernatant was centrifuged, and then separated by decanting. This extract was evaporated (approximately 2days), and reconstituted in 1ml of 100% methanol, following a dilution of 1:31 in 100% methanol (Christianson & Creel 2009).

We performed full-spectrum scans in the *Genesys 10s UV-Vis* spectrophotometer on pure extracts of ethanol and on extracts from faecal samples, measuring optimal density every 1nm from 190nm to 1100nm, focusing on optimal density at 470nm (peak absorption of carotenoids), 666nm (peak absorption of chlorophyll) and at 750nm (correction for turbidity) (Christianson & Creel 2009; Christianson & Creel 2015).

3.2.5) Statistical analyses

The diet composition is expressed in terms of absolute frequency of occurrence (AF) and relative frequency of occurrence (RF) of each plant species consumed of arboreous species, shrub species and herbaceous species. These frequencies are calculated by:

$$AF = \left(n_i / N_f\right) \times 100$$

$$RF = (n_{ei} / N_e) \times 100$$

where n_i is the number of plant fragments of the specie i, N_f is the total number of fragments in the sample, n_{ei} is the number of faeces with plant fragments of the specie i and N_e is the total number of faeces. A high number of different species had AF lower than 1%, as so we decided to gather these species in a group named "Other species". The frequencies of occurrence (AF and RF) were calculated for the different classes (males, females and calves) and for the different seasons (rut, autumn, winter and spring).

Multivariate analyses were performed in order to evaluate differences in the diet composition between the sexes and also other possible relevant factors such as season and age. Multivariate techniques are used because they detect and represent the underlying structure of the data and have the capability to discriminate different groups. These analyses consisted of one ordination method, more specifically principal component analyses (PCA) and also a permutation multivariate analysis of variance (PERMANOVA). These statistical analyses were performed using Canoco 5 and Primer 6+PERMANOVA software.

The species diversity of the diet was evaluated using different diversity indexes, namely through the calculation of species richness (S), Shannon-Weaver's diversity index (Spellerberg & Fedor 2003; Shannon 2001) and Pielou's evenness index (Pielou 1966). Species richness represents the number of different species present in the sample. The Shannon-Weaver's index predicts the diversity of the sample, is a measure of the number of common species (Brewer & Williamson 1994) and is represented by:

$$H' = -\sum p_i \times Ln(p_i)$$

where, $p_i = n_i / N_f$, being n_i the number of plant fragments from specie i; N_f the total number of fragments in the sample. The Pielou's evenness index represents the uniformity of the data and it is calculated by:

$$J' = H' \,/\, H'_{max}$$

where, $H'_{\max} = ln(S)$.

According to Jost (2006), food amplitude ($e^{H'}$) can be interpreted through a transformation on the Shannon index (H') converted in order to represent the actual number of species.

Differences between males and females in terms of diversity (H'), specific richness (S), evenness (J') and food amplitude (dependent variables) throughout seasons were analysed using general linear models. Pairwise comparisons were performed using Boferroni correction.

Schoener index (Schoener 1974) was also performed in order to assess food overlap and it is represented by:

$$O_{jk} = 1 - 1/2 \sum |p_{ij} - p_{ik}|$$

O_{jk} represents the overlapping food between the class j and k, p_{ij} is the proportion of specie *i* in the class j and p_{ik} represents the proportion of specie i in the class k. This index varies between 0 (no food overlap) and 1 (completely food overlap).

Regarding diet quality (carotenoids and chlorophyll, dependent variables), general linear models were used to evaluate the differences between sexes and seasons (independent variables).

The statistical analyses were performed using IBM.SPSS, version 22. All statistical analyses were considered significant when $p < 0.05$. The results are presented as estimated $mean \pm standard\ error\ (SE)$.

3.3) Results

3.3.1) Diet composition

Globally, the shrub species were the most consumed group (54.65%), followed by the monocotyledons (28.80%), arboreous (8.26%) and dicotyledons species (2.45%). *Pterospartum tridentatum* is the most consumed shrub species ($29.58\ \%$), and together with the *Ulex minor* ($8.01\ \%$) are responsible for the high percentage of shrub species found. The most representative arboreous species was *Acacia melanoxylon* (3.17%). The herbaceous

group stood out (31.25%), with monocots with a higher representativeness than dicots, as showed by the species with higher values, the *Hordeum murinum* (15.51%) and the *Athyrium felix-femina* (1.31%), respectively. Besides that, the "Species NI" group had a very low absolute frequency of occurrence (0.26%) which indicate a high rate of identification of the fragments.

The absolute and relative frequencies of occurrence were calculated for the different sexes (Table 1) and the rank of the most consumed groups were similar to the global diet of the red deer. However, males consumed more arboreous species than females and calves (Fig. 9). The opposite patter was observed for shrub species (Fig. 9).

Table 1: Diet composition of males ($N = 51$), females ($N = 64$) and calves ($N = 26$) of red deer in terms of absolute frequency of occurrence (AF) and relative frequency of occurrence (RF).

	Males		**Females**		**Calves**	
	AF(%)	RF(%)	AF(%)	RF(%)	AF(%)	RF(%)
Arboreous species	**12.44**	**98.04**	**6.34**	**95.31**	**4.79**	**100**
Acacia melanoxylon	5.72	58.82	2.26	45.31	0.42	23.08
Castanea sativa	2.08	52.94	0.62	23.44	0.69	26.92
Chamaecyparis lawsoniana	1.3	64.71	1.33	81.25	1.63	92.31
Fraxinus sp.	1.47	35.29	0.77	20.31	1.23	23.08
Laurus nobilis	1.87	47.06	1.37	23.44	0.81	26.92
Herbaceous species	**30.25**	**100**	**32.52**	**100**	**30.08**	**100**
-Dicots	**2.69**	**68.63**	**2.48**	**75.00**	**1.9**	**61.54**
Athyrium filix-femina	1.25	66.67	1.45	60.94	1.1	61.54
Omphalodes nitida	1.44	47.06	1.03	67.19	0.81	57.69
-Monocots	**31.59**	**100**	**29.07**	**100**	**25.13**	**100**
Agrostis castellana	3.48	74.51	1.93	73.44	1.04	53.85
Dactylis glomerata	7.41	93.75	8.27	100	4.71	84.31
Gramineae NI	3.55	92.19	4.04	88.46	5.58	80.39
Hordeum murinum	17.15	93.75	14.83	92.31	13.8	94.12
Shrub species	**50.62**	**100**	**55.79**	**100**	**59.75**	**100**
Cytisus striatus	8.93	86.27	5.48	89.06	5.29	76.92
Erica arborea	1.82	72.55	2.97	81.25	2.88	88.46
Erica australis	2.5	76.47	2.96	85.94	3.46	88.46
Erica umbellata	1.44	64.71	2.73	67.19	2.19	69.23
Genista triacanthos	1.61	70.59	1.59	50	1.9	50
Pterospartum tridentatum	21.75	92.16	32.62	100	37.46	100
Rubus ulmifolius	1.83	45.1	0.78	45.31	0.62	34.62
Ulex minor	10.74	90.2	6.67	84.38	5.94	76.92
Other Species	**6.41**	**100**	**5.04**	**95.31**	**5.31**	**96.15**
Species NI	**0.27**	**45.1**	**0.32**	**43.75**	**0.08**	**15.38**

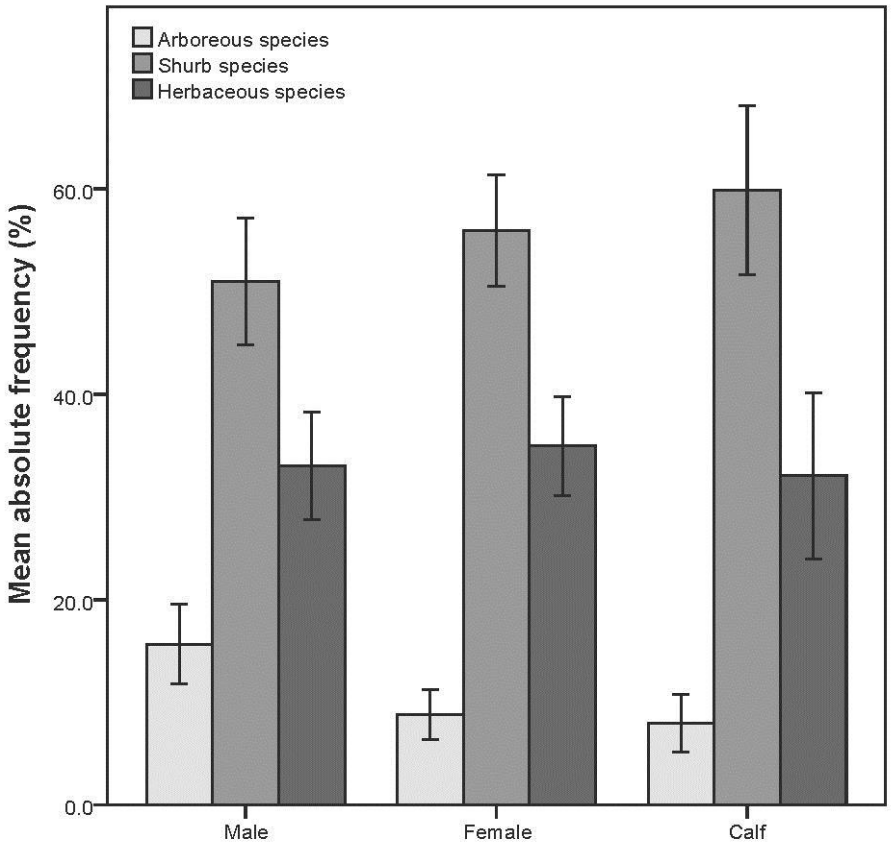


Fig 9: Mean absolute frequency of occurrence of the different groups of plant species present on the diet of red deer males ($N = 51$), females ($N = 64$) and calves ($N = 26$).

Analysing the mean absolute frequency of occurrence of the three main plant groups of each sex in each season (Table 2), our results show that males eaten more arboreous species in all seasons. Shrub species were more consumed by females in all seasons, except for spring. In the spring season, the bigger difference between males and females occur in the herbaceous species, where females eat approximately the double comparing with males (Table 2).

Table 2: Mean absolute frequency of occurrence of the three main plant groups consumed by red males ($N = 51$), females ($N = 64$) and calves ($N = 26$) in the different seasons.

		Males	Females	Calves
Rut	Arboreous species	21.2 ± 5.1	16.6 ± 3.4	11.9 ± 5.3
	Shrub species	31.9 ± 4.2	39.5 ± 5.1	47.1 ± 4.6
	Herbaceous species	46.5 ± 3.6	43.3 ± 4.9	40.9 ± 7.9
Autumn	Arboreous species	16.1 ± 2.5	6.9 ± 1.5	4.8 ± 1.0
	Shrub species	56.8 ± 5.1	63.7 ± 3.4	68.5 ± 6.9
	Herbaceous species	26.9 ± 4.6	29.0 ± 3.2	26.6 ± 7.0
Winter	Arboreous species	12.6 ± 2.8	4.3 ± 0.8	7.6 ± 2.2
	Shrub species	67.4 ± 4.5	74.1 ± 3.4	74.5 ± 3.3
	Herbaceous species	19.9 ± 3.92	21.5 ± 3.4	17.8 ± 3.1
Spring	Arboreous species	8.5 ± 1.4	7.5 ± 2.7	9.6 ± 2.6
	Shrub species	53.9 ± 6.2	38.6 ± 4.1	35.6 ± 5.7
	Herbaceous species	37.3 ± 5.8	53.7 ± 4.2	54.7 ± 5.6

Our results showed significant differences between sexes $\left(pseudo - F_{(2,129)} = 3.174;\ p = 0.001\right)$ in terms of diet composition. In terms of differences between the sexes, diet composition of males was significantly different from females $(t = 2.068;\ p = 0.005)$ and calves $(t = 2.091;\ p = 0.002)$. On the other hand, females and calves are not statistically different $(t = 0.775;\ p = 0.657)$.

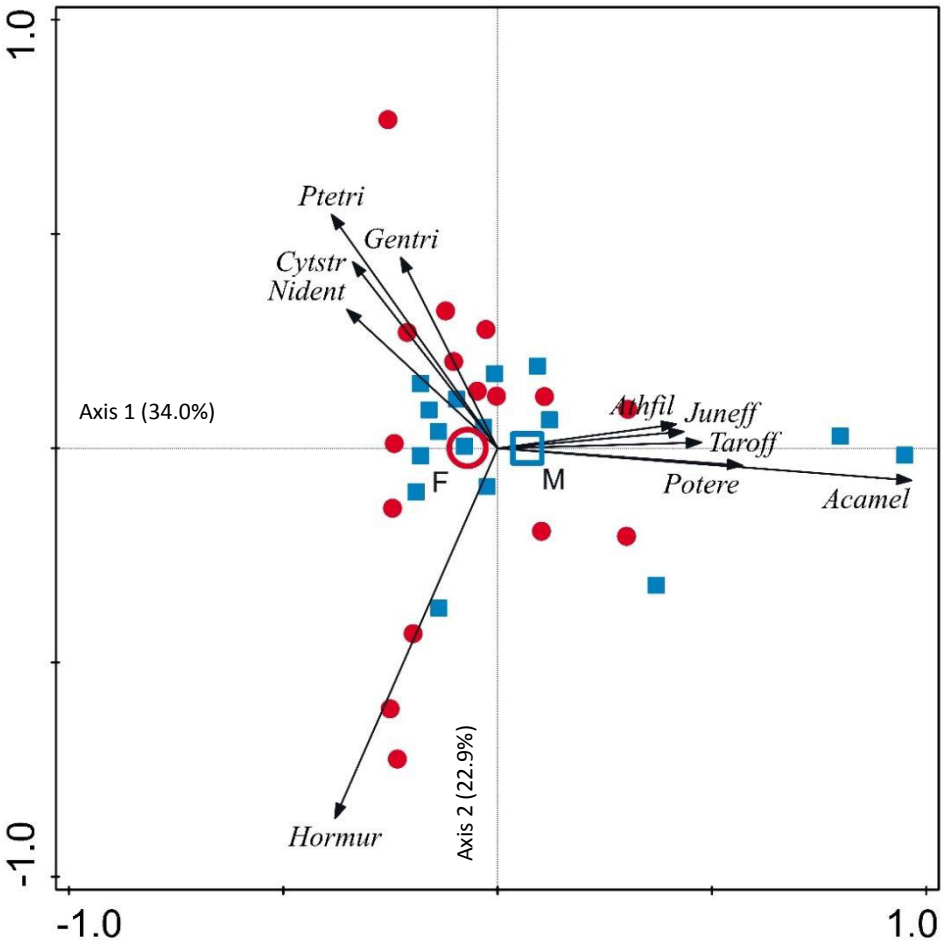
Regarding age classes, no differences were detected due to this factor $\left(pseudo - F_{(1,121)} = 1.912;\ p = 0.095\right)$. Also in the relation between age and the other factors (sex and season) was observed that there were not statistically significant differences.

Our results demonstrate significantly differences in the seasons $\left(pseudo - F_{(3,129)} = 10.708;\ p = 0.001\right)$. Indeed, when we tested the comparisons between seasons, results show that significant differences were obtained in all pairwise comparisons.

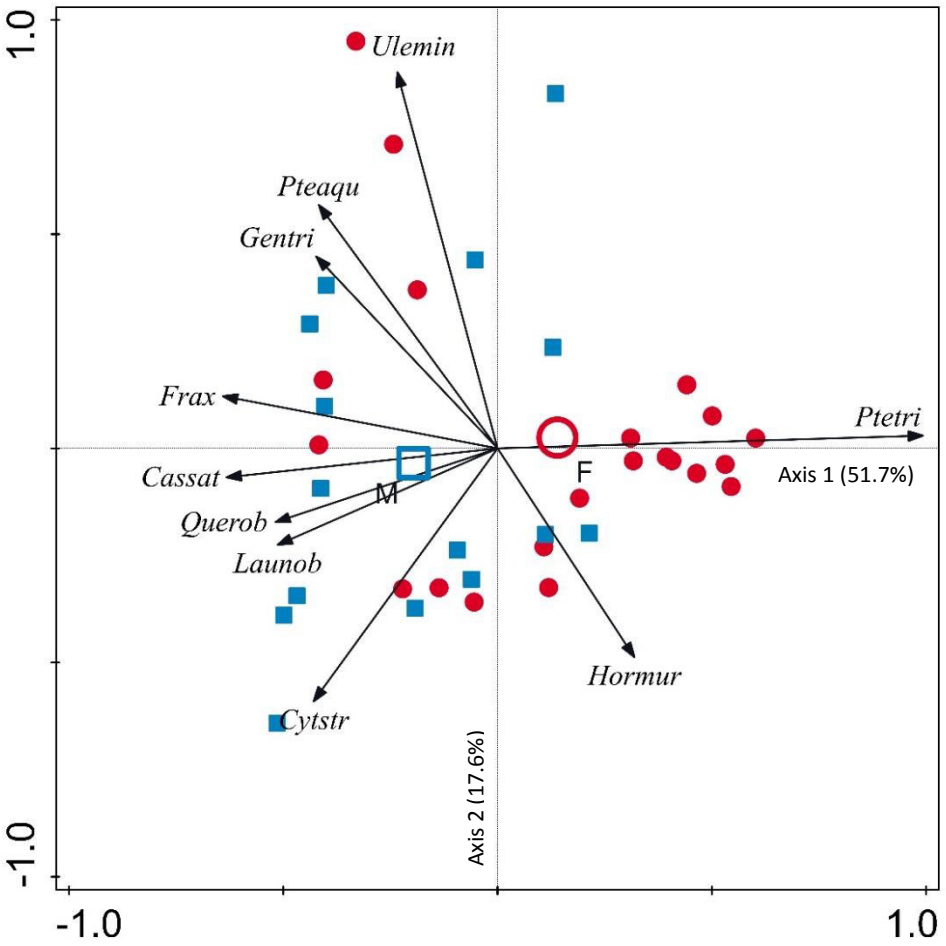
When analysing the influence of the seasons in the diet of both sexes, significant differences were found. In the rut season, was not verified significant differences between males and females $(t = 1.175;\ p = 0.220)$, but is possible to observe the relative importance of *Acacia melanoxylon* and *Hordeum murinum* in males and females, respectively (Fig. 10 a). In Autumn, it is notice a statistically significant difference between males and females $(t = 2.248;\ p = 0.003)$, with males consuming comparatively more *Quercus robur*, *Castanea sativa* and *Cytisus*

striatus than females, while females consume more *Pterospartum tridentatum* than males (Fig. 10 b). In the winter season, the diet composition was almost overlapped between the sexes ($t = 0.855$; $p = 0.519$), with males eating a little more *Eucalyptus globulus* and *Ulex minor* than females and females more *Pterospartum tridentatum* than males (Fig. 10 c). Significantly differences between the sexes ($t = 1.950$; $p = 0.007$) are also showed in spring season, where *Genista triacanthos* and *Ulex minor* for males, and *Erica* genus and Gramineae for females, are the species that contribute more to differentiate the sexes (Fig. 10 d).

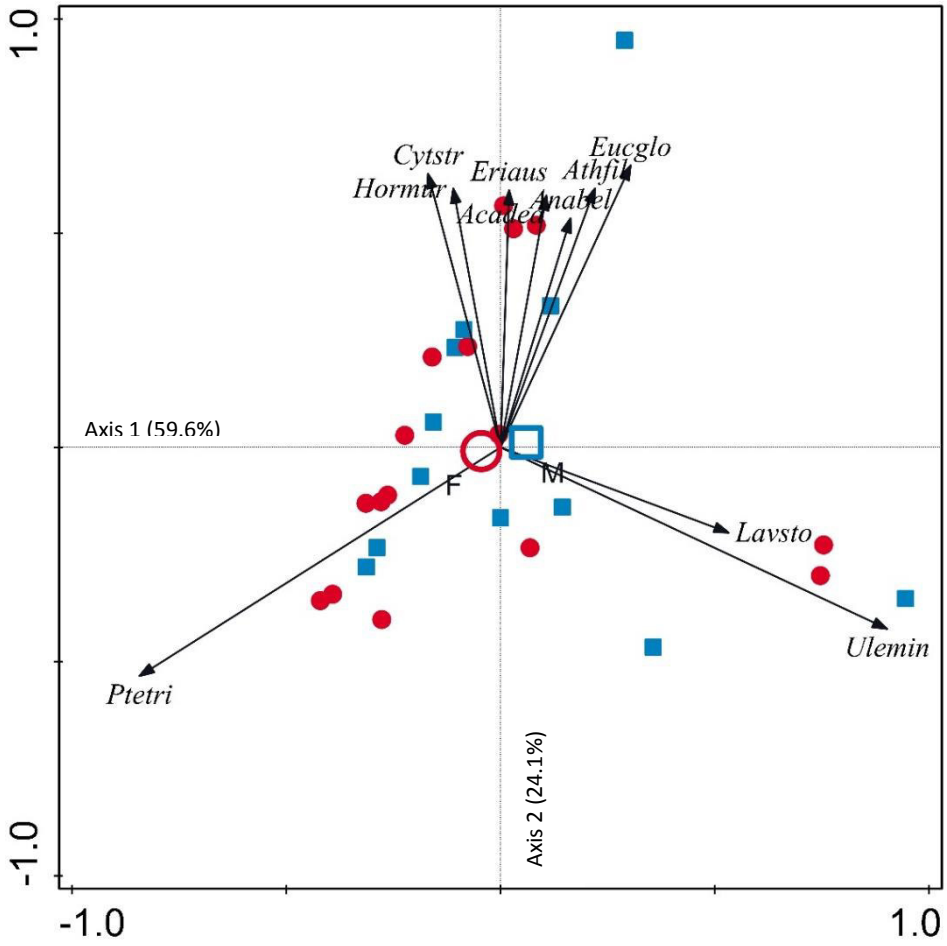
a)



b)


Ulemin
Pteaqu
Gentri
Frax
Cassat
Querob
Launob
Cytstr
Hormur
Ptetri
F
M
Axis 1 (51.7%)
Axis 2 (17.6%)
1.0
-1.0
-1.0
1.0

c)


Cytstr
Eriaus
Eucglo
Athfil
Hormur
Anabel
Acadec
Axis 1 (59.6%)
F
M
Lavsto
Ulemin
Ptetri
Axis 2 (24.1%)
1.0
-1.0
-1.0
1.0

d)

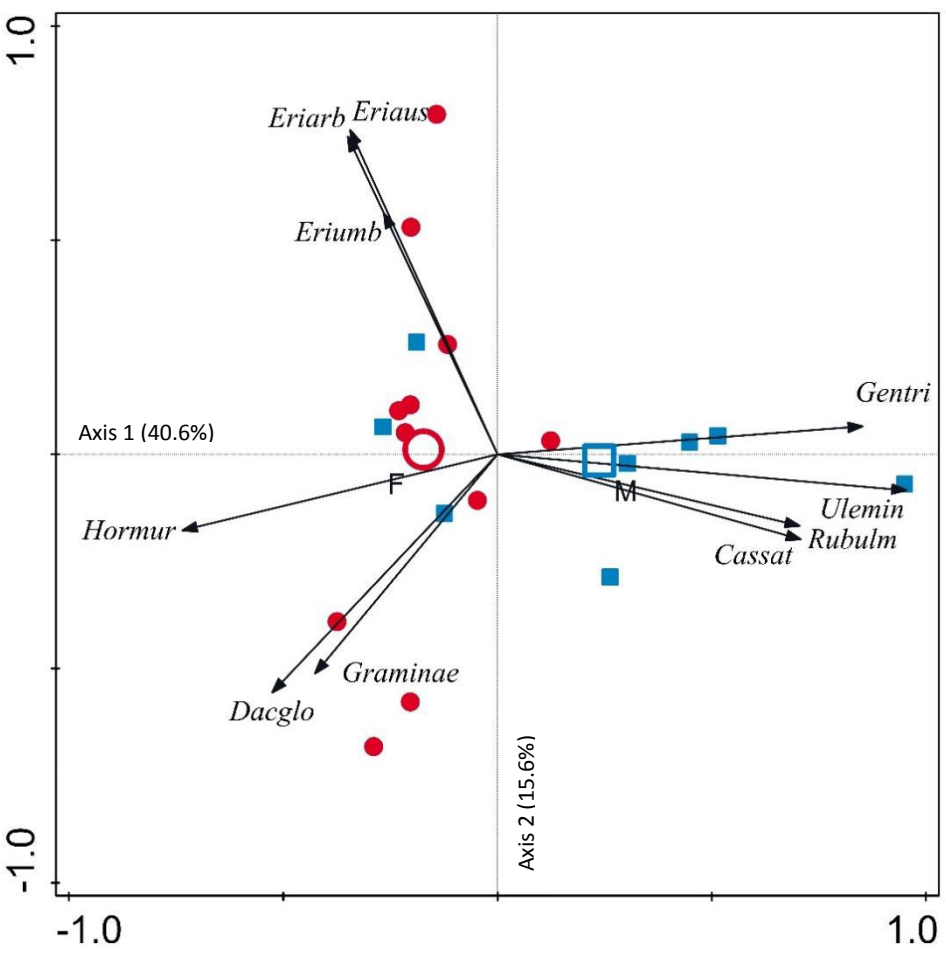


Fig 10: PCA biplot for a) rut, b) autumn, c) winter and d) spring seasons, showing the differences between sexes. Red circles represent the females ($N = 64$) and blue squares the males ($N = 51$).

3.3.2) Diet diversity

In terms of diet diversity analysed by the Shannon-Weaver index (H') (Fig. 11), our results demonstrate significantly differences between seasons ($p < 0.001$). When analysing the pairwise comparisons, significant differences were found between winter (1.60 ± 0.069) and autumn (2.00 ± 0.061), or rut (2.13 ± 0.064) or spring (2.04 ± 0.084), with $p < 0.001$ for all. Considering sex, there were differences between diet diversity of both sexes ($p = 0.038$). From our results it is possible to observe that males (2.01 ± 0.052) and females (1.87 ± 0.046) differ in their diet diversity. When analysing the sexes within the seasons, those values were not significant different ($p = 0.578$).

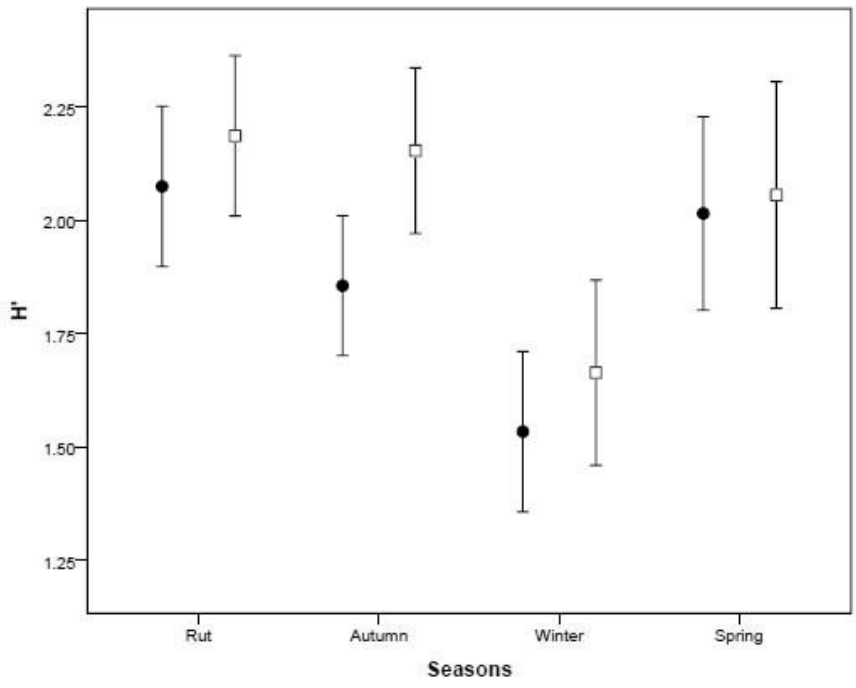


Fig 11: Mean Shannon-Weaver diversity index by season between males (squares, $N = 51$) and females (black circles, $N = 64$).

In terms of species richness (Fig. 12), our results demonstrate that the number of species consumed vary significantly between seasons ($p = 0.001$). From pairwise comparisons, differences between winter and autumn ($p = 0.007$) and winter and rut ($p = 0.004$) were found. These differences are also showed by the lower values of species richness obtained in

winter (15.55 ± 0.657) when compared with autumn (18.41 ± 0.582) or rut (18.59 ± 0.609). There were not significant differences between males and females ($p = 0.168$), neither when analysing the diet diversity of each sex within the seasons ($p = 0.560$).

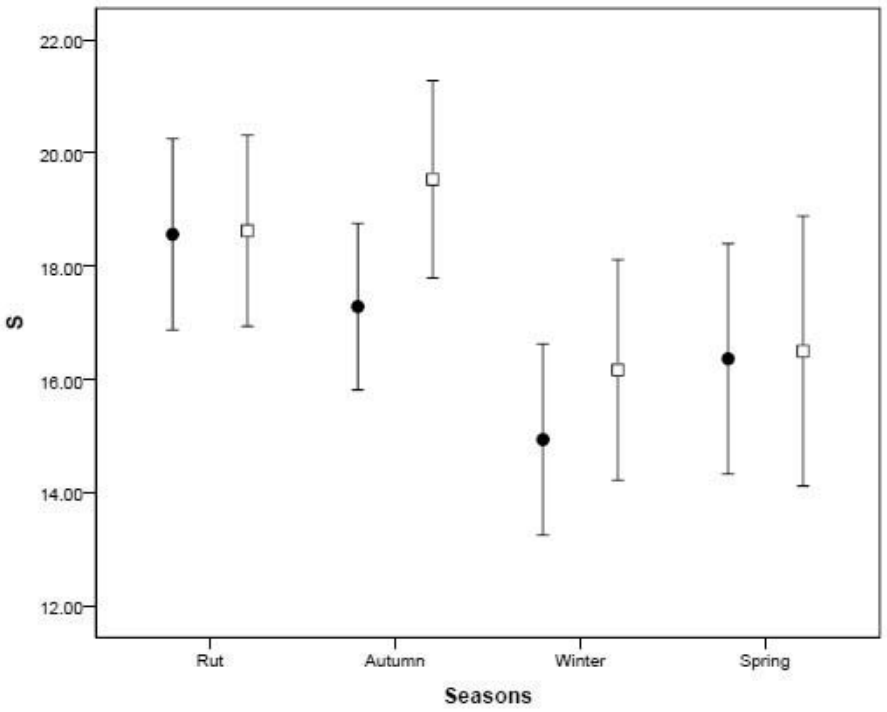


Fig 12: Mean Species richness by season between males (squares, $N = 51$) and females (black circles, $N = 64$).

In terms of diet homogeneity analysed by the Pielou index (Fig. 13), significant differences between seasons ($p = 0.000$) were found, in which the winter shows lower values of evenness (0.59 ± 0.018) compared with the other seasons (autumn = 0.69 ± 0.016, rut = 0.73 ± 0.017 and spring = 0.73 ± 0.022). These values are corroborated by the results of pairwise comparison where $p < 0.001$ for comparisons between winter and all the other seasons. Considering sex, there were also significant differences between the sexes ($p = 0.041$). Indeed, males had a higher value (0.70 ± 0.014) than females (0.66 ± 0.012) in terms of evenness. Regarding the analyses of sex within each season, significant differences were not found ($p = 0.691$).

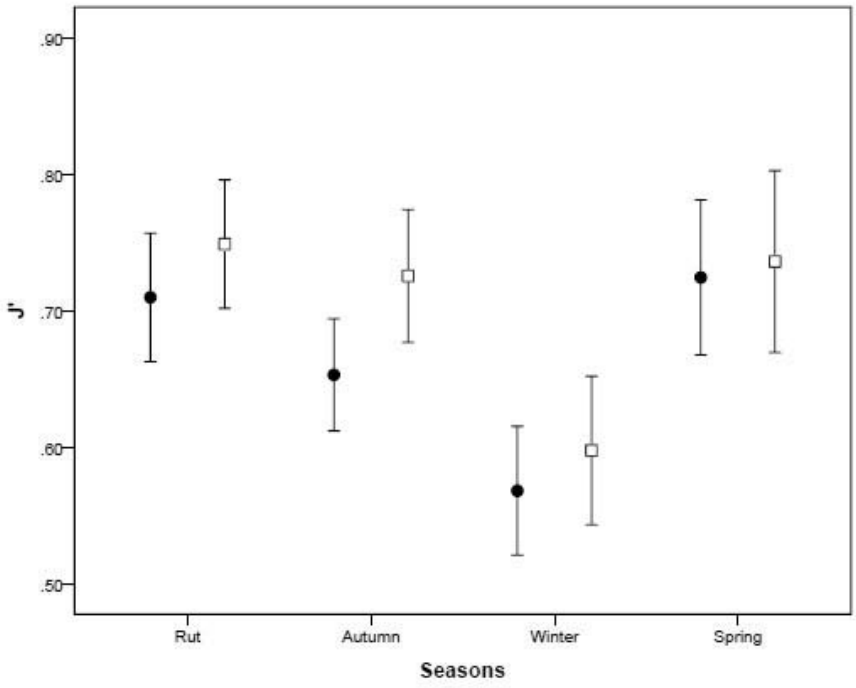


Fig 13: Mean Pielou evenness index by season between males (squares, $N = 51$) and females (black circles, $N = 64$).

The food amplitude (Fig. 14) between males and females showed significant differences between the seasons ($p < 0.001$). The winter is the season with lower values (5.25 ± 0.502) comparatively with autumn (8.12 ± 0.044), rut (8.95 ± 0.464) or spring (7.85 ± 0.611). Significantly differences ($p < 0.001$) between winter and autumn or rut, and also spring (p=0.006) were observed in the pairwise comparison. Moreover, there were no significant differences between males and females ($p = 0.061$) or for them in each season ($p = 0.637$).

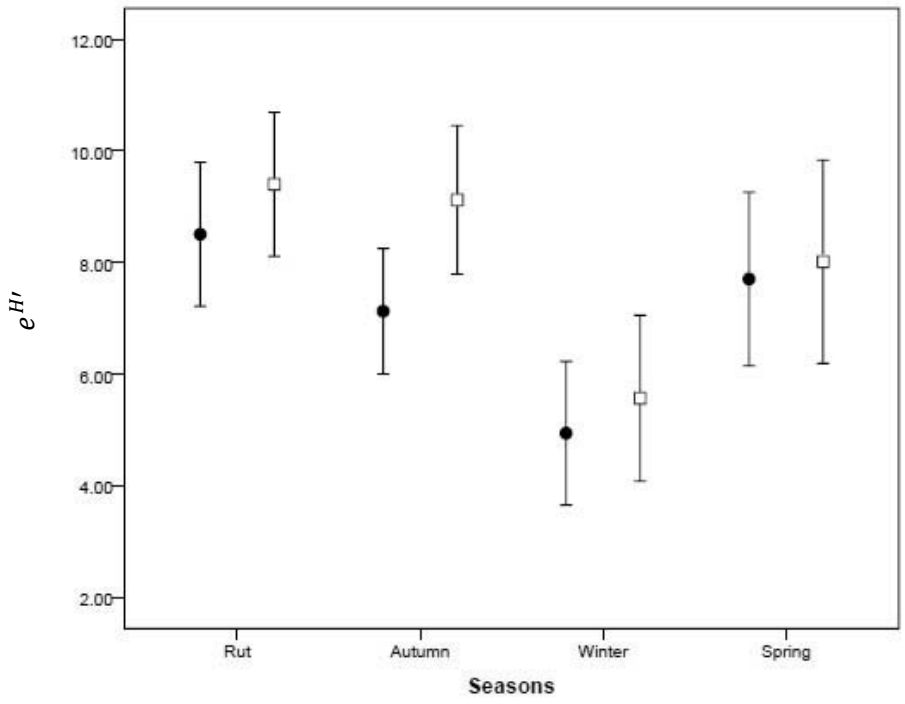


Fig 14: Mean food amplitude index by season between males (squares, $N = 51$) and females (black circles, $N = 64$).

Analysing the Schoener index, there was no completely overlap between the sexes. Our results showed that in rut and winter the food overlap between males and females is higher. On the other hand, in spring and autumn food overlap between the sexes decreases.

Table 3: Schoener index representing food overlap between males and females

	Schoener index	Confidence Interval[1]
Rut	0.810	0.764 - 0.897
Autumn	0.687	0.633 - 0.795
Winter	0.883	0.713 - 0.947
Spring	0.661	0.468 - 0.820

[1]calculated using bootstrap

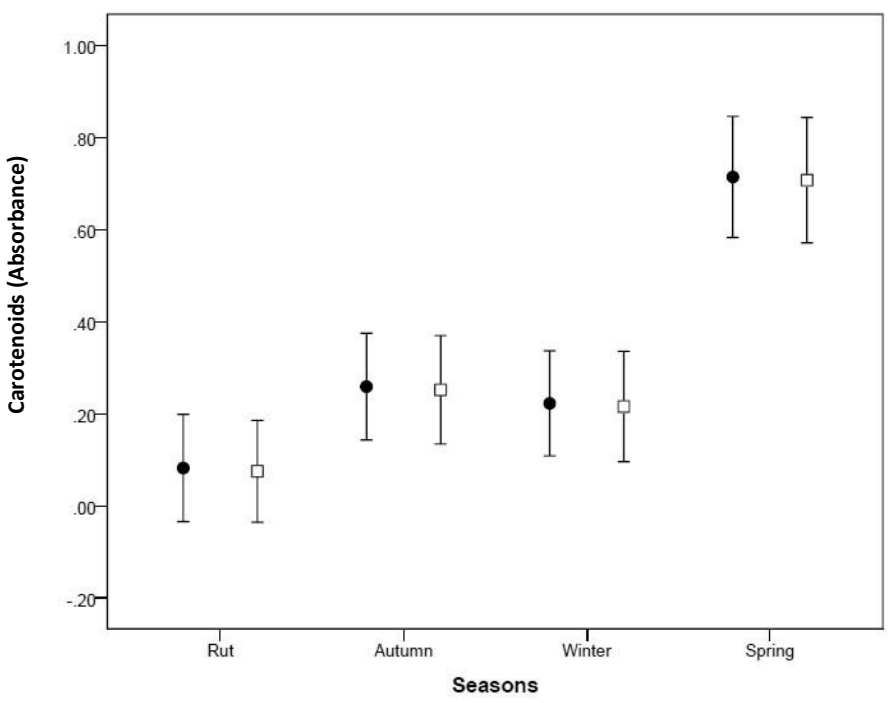
At a general level, from our results, regarding diversity, species richness, evenness and food amplitude there were always significant differences between the seasons. Significant

differences between males and females were only verified concerning Shannon's diversity and Pielou's evenness.

3.3.3) Diet quality

According to Christianson & Creel (2015), concentrations of photosynthetic pigments, meaning carotenoids and chlorophylls, are related with forage quality. Regarding carotenoids and chlorophylls (Fig. 15), in both cases there were significant differences between seasons ($p = 0.000$ for both pigments). The rut season showed lower values for both carotenoids and chlorophylls (0.08 ± 0.051 and 0.19 ± 0.034, respectively) compared with the other seasons. Spring presented with higher values of carotenoids and chlorophylls (0.71 ± 0.062 and 0.43 ± 0.041, respectively). Concerning the comparison of males and females, no significant differences between the sexes ($p = 0.899$ and $p = 0.271$ for carotenoids and chlorophylls respectively) were found.

a)



b)

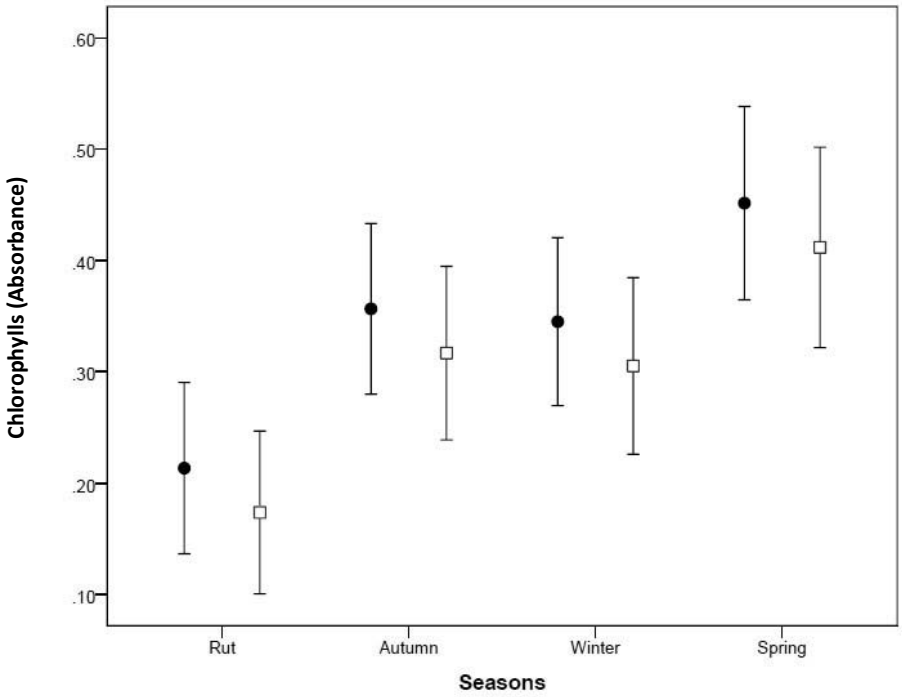


Fig 15: Concentration of photosynthetic pigments, a) carotenoids (optimal density at $470nm$) and b) chlorophyll (optimal density at $666nm$), in red deer faeces of males and females in each season. Squares represent males ($N = 51$) and black circles the females ($N = 64$).

3.4) Discussion

3.4.1) Feeding behaviour

Red deer diet is mainly constituted by shrub species, as also described by (Milne et al. 1978; Suter et al. 2004; Ramirez et al. 1996). Our results exhibited, in this plant group, a higher consumption of *Pterospartum tridentatum,* followed by *Ulex minor*, explain by their high availability in the whole study area. Another important group for red deer is the monocotyledons, with a higher percentage of Gramineae being consumed, more specifically *Hordeum murinum*, as also shown by Holechek (1981); Dumont et al. (2005); Sanders et al. (1980) and Cortez (2010). Although dicotyledons were in general not very consumed by red deer (Bugalho et al. 2001; Suter et al. 2004; Merril et al. 1995), the diversity of species of this group that was detected was considerably high, probably because of the florist diversity of the study area. This result may be related with accidental ingestion, and not a real selection of these species. The group with the lowest representability in the diet was the arboreous species, being the *Acacia melanoxylon* the most consumed species.

Red deer is considered as an intermediate feeder (Hofmann 1989), and its classification is supported by our results. In the Lousã Mountain, red deer behaves as a "grazer", consuming grasses, and also as a "browser", feeding on shrub and arboreous species (Gebert & Verheyden-Tixier 2001; Bugalho et al. 2001; Szemethy et al. 2003; Bugalho & Milne 2003; Ruckstuhl & Neuhaus 2005; Dumont et al. 2005; Alves 2013).

Regarding the temporal patterns on diet composition, our results showed significant differences in the food items present in the faeces. These differences are mainly related with plants' phenology, but can also reflect the availability of prefer species. In seasons where the availability of resources is higher in the Mediterranean region (*i.e.* Spring), the diversity of species eaten is lower, indicating that animals are feeding on their preferred items. In limiting seasons, like Summer and Rut, red deer makes use of a larger variety of food items to accomplished its energetic requirements.

Regarding methodologic options, to determine the diet of red deer, the microhistological technique was used. This technique assumes that plant epidermises are resistant to the digestive processes, with the maintenance of the anatomy when excreted, and also that the excreted amount of each plant epidermis is proportional to that ingested (Maia et al. 2003). This technique is widely used, providing good overall results. However, it has also limitations like the time to perform the analysis (Holechek & Vavra 1981; Holechek et al. 1982; Carrière 2002; Maia et al. 2003; Ahmed et al. 2015; Dove et al. 1995). However, the main concern is the possible overestimation of fibrous plants and underestimation of highly digestible herbaceous species (Holechek et al. 1982; Ramirez et al. 1996; Dumont et al. 2005). Some authors, consider that the digestive process has influence on the recognition and identification of the fragments, because the epidermises can suffer differentiated digestion of tissues according to the anatomic and chemical characteristics of the plants (Sparks & Malechek 1968; Sanders et al. 1980; Holechek et al. 1982; Butet 1985; Dove et al. 1995). However, Bauer et al. (2005) concluded that the differential digestion happens only in a small percentage of samples and the effects are only minor, not affecting significantly and not compromising the results (Dearden et al. 1975; Bauer et al. 2005). Moreover, if the number of the unidentifiable fragments was higher than 10%, it would mean that the reference collection did not have the necessary species to enable the correct identification of the fragments, being necessary to reinforce it with more plant species. Since the percentage of not identified species is lower than 1%, we can conclude the suitability of the reference collection and dichotomous key elaborated in the first phase of the project, and the suitability of this method to evaluate the diet composition of red deer in the Lousã Mountain. Regarding the quantification of the pigments of plant material found in faeces, the

method has three main advantages 1) pigments are indigestible, 2) are not confounded with metabolic by-products in faeces and 3) are easily extracted from faeces (Reid et al. 1949; Christianson & Creel 2015). Christianson & Creel (2015) compared the analyses of faecal nitrogen with faecal chlorophyll and concluded that faecal chlorophyll had a better fit to their model than faecal nitrogen (analysed at an independent laboratory). According to their results, concentrations of photosynthetic pigments were correlated with forage quality (*i.e.*, digestibility, energy content, neutral detergent fibre and nitrogen content).

3.4.2 Evaluating forage selection hypothesis

According to the FSH, the sexes should have differences in their foraging behaviour. Males would ingest higher biomass but lower quality forage, choosing for fibrous plants, because they are good at digesting them due to the large rumen. On the other hand, females would ingest lower quantity of food, and choose habitats with higher forage quality, selecting species with high nitrogen contents because of their lower efficiency in digesting (Bowyer 2004).

Our results demonstrate that those differences between the sexes occurred in terms of diet composition and proportions, and also in terms of diversity (Shannon-Weaver and evenness indexes), where red deer males and females showed statistically significant differences in their diet spectrums. This fact could be related with body size dimorphism and different gut capacity, leading to different nutritional requirements between the sexes (Illius & Gordon 1987; Ruckstuhl & Neuhaus 2000; Bugalho et al. 2001). Those differences could lead to different food selection and then habitat segregation (Ruckstuhl & Neuhaus 2000; Barboza & Bowyer 2000). Different body size dimorphism could explain those differences in diet composition, since males and females show different strategies, *i.e.* males seem to be more adapted to browsing (because they are bigger) and females for grazing (Bugalho et al. 2001). In fact, the proportion of arboreous species consumed was higher for males than females, supporting the adaptation previously mentioned.

Concerning the food overlap between the sexes, our results showed that in the rut, when the sexes are aggregated, the food overlap index between males and females is higher, which is in agreement with the expected results. In spring, food overlap decreases, and this may be related with the sexual segregation observed in this season (Alves et al. 2013). In autumn season, when sexes are randomly associated our results demonstrate an intermediate food overlap. In winter, there was a high value of food overlap between males and females, although in terms of sexual

segregation patterns, this was an unexpected result. This may be the result of a lower plant diversity but more probably due to the high abundance of preferred species like heathers and other shrubs.

Seasonal differences were also shown in our results. Spring and autumn were the seasons in which higher differences between males and females regarding the composition of the diet were detected. This fact may be related with the different requirements of both sexes in these specific times of the year. Autumn is right after rut season, and the sexes have to recover weight and fat reserves, while spring and early summer it is a delicate season for females because they are at the end of gestation or beginning of lactation (Main et al. 1996; Bugalho & Milne 2003). On the other hand, considering diversity, winter season was the most different season from all the others where the diversity of diet consumed was lower. Although the consumed biomass of a specific plant increases in this season, these result could be related with low plant diversity available. Similar differences were also found in other studies (Dumont et al. 2005). At a general level, our results suggest that feeding ecology is influenced by seasonal changes, related with plant availability and phenology (Garcia-Gonzalez & Cuartas 1989; Prokešová 2004; Dumont et al. 2005).

In herbivore's communities, the higher consumption of one plant species rather than the other has nutritional effects on the animal (Dove et al. 1995). Regarding those effects and considering FSH, it was expected that males and females had differences in quality of the diet. We analysed the photosynthetic pigments (carotenoids and chlorophylls) present in the faeces, which according to Christianson & Creel (2015) are related with the food quality ingested. Faecal carotenoids and chlorophyll could represent how the animal concentrates these pigments in their faeces, which can be measured because they are resistant to digestion and sensitive to light absorption (Christianson & Creel 2009). However, contrarily to our predictions the results showed that there were no significant differences between the sexes, either for carotenoids or chlorophyll excreted in faeces. Those results could be related with differences in the forage absorption and excretion between males and females because they have different digestive systems and gut capacities. Besides that, forage quality varied significantly between the seasons, and as expected spring arises as the season with higher-quality food (as also showed by Christianson & Creel (2015)). Photosynthetic pigments in faeces could represent the variation in productivity and quality (Christianson & Creel 2015), so our results are in agreement with our expectations in terms of seasonal patterns.

Our results also demonstrate that between females and calves, there were no significantly differences in terms of diet composition, which was expected because of the social organization of this species, *i.e.* matriarchal groups (Alves 2013). Indeed, considering feeding efficiency, it is predictable that females and calves to remain in the same feeding areas during all year, exhibiting both similar patterns in diet composition and species abundance (Ruckstuhl & Neuhaus 2005). The motivations that lead females to remain with other females, and sub-adult males to leave their natal groups may be related to the nutritional benefits of remaining or leaving. So, one major question in sexual segregation is why young males start to segregate themselves from females (Ruckstuhl & Neuhaus 2005). Based in our results, this remains a question without a conclusive answer. Energetic requirements may help to explain sexual segregation, but the observed results did not show enough differences between foraging behaviour of males and females to fully support the assumptions of FSH (also showed by Miquelle et al. (1992); Bonenfant et al. (2004)). As so, and notwithstanding the importance of habitat segregation and foraging behaviour to sexual segregation, it is important keep researching to understand which other factors may be behind this phenomenon.

According to the results of Alves et al. (2013), sexual segregation in red deer is characterized by the differential use of space by adult males and sexually active females resulting from different strategies all year except in the rutting season, when the sexes are aggregated. As showed by the results of Alves et al. (2013), sexual segregation could not be explained by one single hypotheses. Their results demonstrate that explaining sexual segregation based only in differential use of habitat by males or females was not possible, because they could not assume that males use lower quality habitats then females. With the conclusions of our study, in which there were not found statistically differences in forage quality between males and females, it is possible to assume that sexual segregation is not only due to differences in the habitat use or forage ecology.

So, answering the question “Sexual segregation in red deer: a question of food?”, although males and females present different functionality categories as consumers (Ruckstuhl & Neuhaus 2005), from our results, these differences are not sufficient to explain all patterns of sexual segregation exhibit by this population, and more factors should also contribute to this phenomenon.

Chapter IV- General conclusions

Microhistological technique became the most used indirect method to assess botanical content of diet of herbivores. This technique is highlighted from the others because it is inexpensive, do not require the killing of the animal and allow the identification of each plant species consumed (Sanders et al. 1980; Holechek 1982). This technique is based on the identification of the fragments of plants present in the faeces. To make identification of those fragments, a previously reference collection of epidermis (Annex 1) is essential (Sanders et al. 1980; Holechek 1982; Szemethy et al. 2003). Aiming to turn this technique more accurate and reliable, besides the reference collection, a dichotomous key (Annex 2) proved to be a useful tool (Ahmed et al. 2015), in combination with the reference collection of epidermis, it facilitates the identification process. Those two approaches are essential for future works, because they can save time allowing an easier identification of the epidermis.

Identifying the plant fragments present in faeces of red deer showed to be a time consuming procedure. In order to overcome this disadvantage of the microhistological technique, accurate analyses of sampling effort according to the objectives of each study are essential. Sampling effort defines the number of fragments needed to represent a determinate percentage of the sample (Shiu & Lee 2003; Gotelli & Colwell 2011). Our results demonstrate that with a total of 200 fragments for each faecal sample, it is possible to obtain a good compromise between precision and cost, providing 95% of the total species richness. Therefore, assess this type of analyses before applying the microhistological technique could be a good strategy to save time and effort.

The analysis of the diet of red deer is important to assess differences in forage ecology of males and females and link them to sexual segregation. Sexual segregation is a complex phenomenon for which there are many answering questions and lack of consistent results (Barboza & Bowyer 2000). This phenomenon can be influencing by different factors and is explained by two components: habitat segregation and social segregation. To explain each component, different hypotheses were postulated by different authors, and this study the main focus was to evaluate the assumptions of one of these hypotheses, the forage selection hypotheses. Our results of foraging behaviour of red deer *Cervus elaphus* showed that there the differences obtained were not enough to explain the patterns of sexual segregation of this wild population.

These facts lead us to assume that there are several factors promoting sexual segregation patterns (Bonenfant et al. 2004; Alves et al. 2013), and that feeding preferences and foraging behavior and only two more factors to consider when studying this phenomenon. Based on the

previous study (Alves et al. 2013) and in the present study, future works should be directed to the social component of sexual segregation, evaluating the activity budget hypothesis and the dispersing tactics employed by sub-adult males and females just after reaching adulthood.

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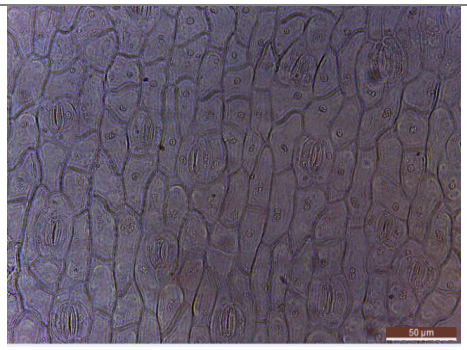
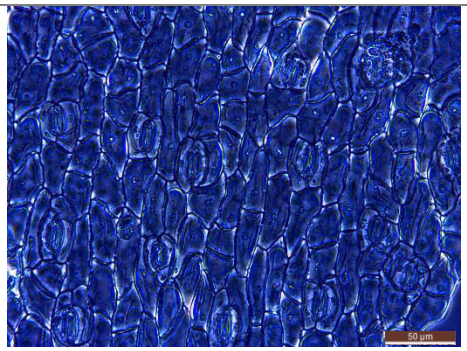
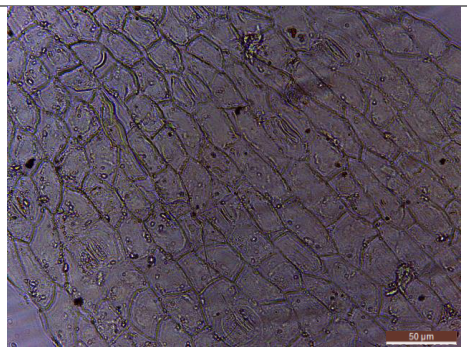
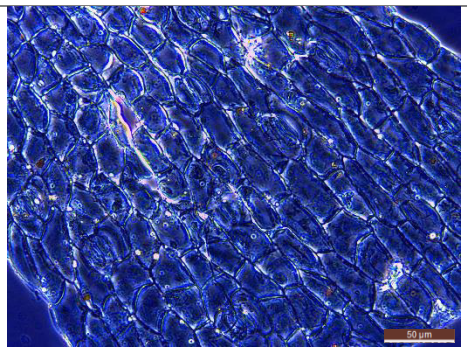
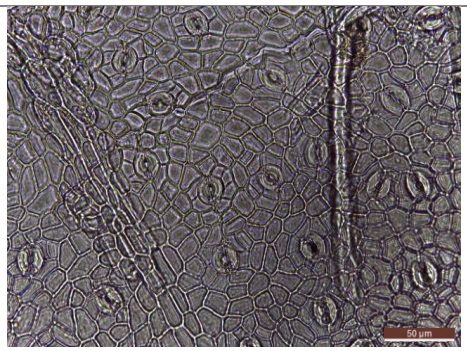
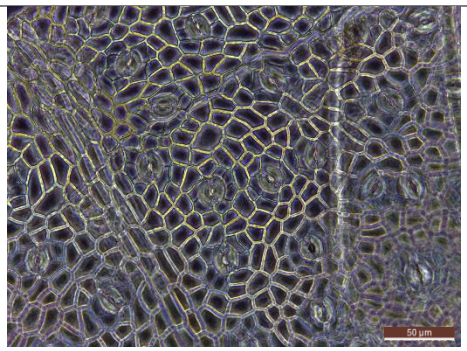
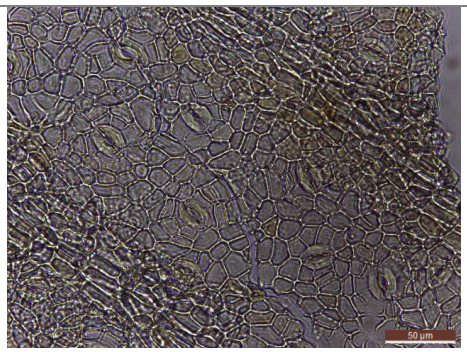
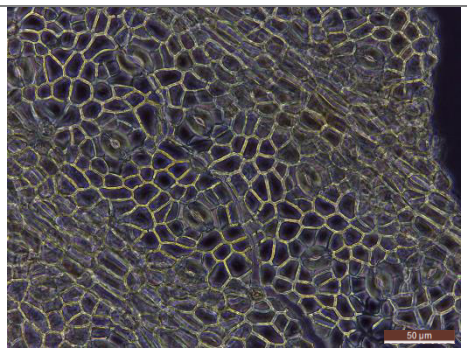
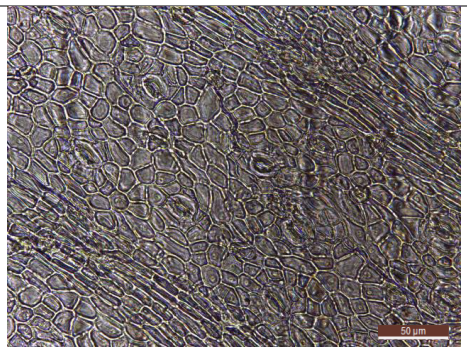
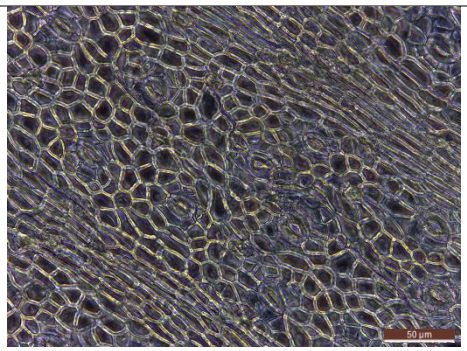
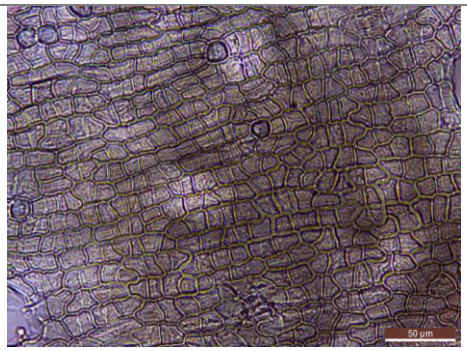
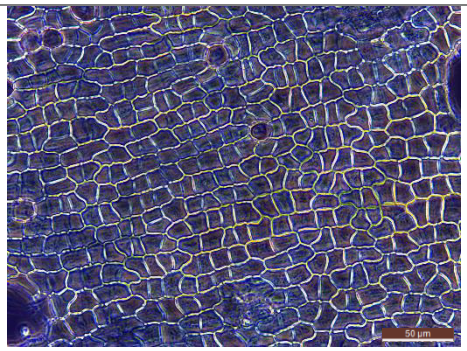
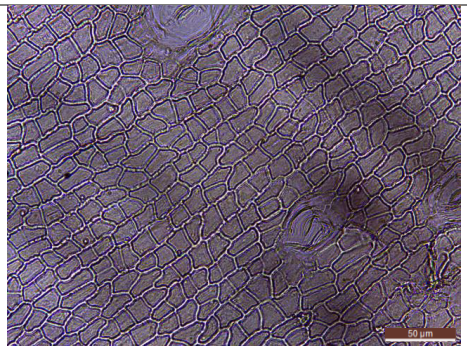
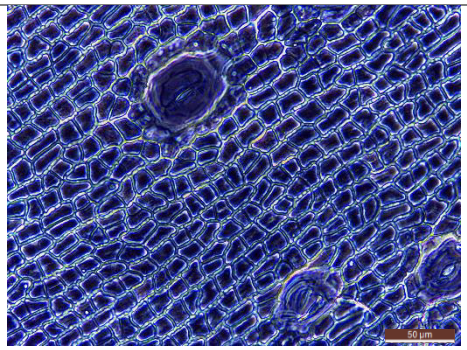
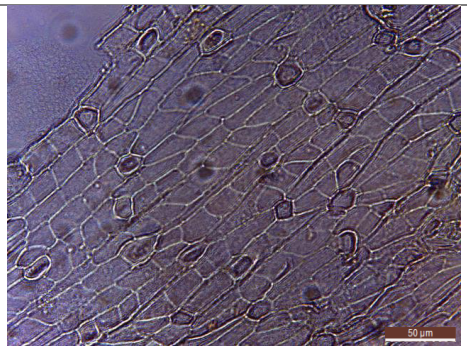
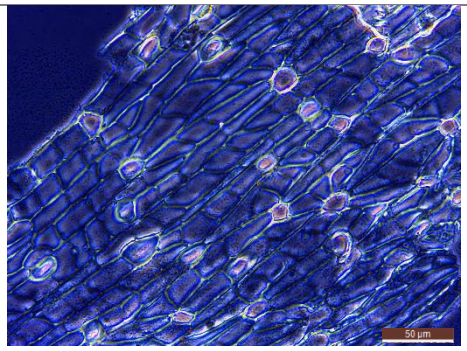
Annex 1

List of species from Lousã Mountain present in the reference collection

Arboreous species	Shrub species	Herbaceous species	
		Dicotyledons	**Monocotyledons**
Acacia dealbata	*Cistus monspeliensis*	*Anarrhinum bellidifolium*	*Agrostis castellana*
Acacia melanoxylon	*Cytisus striatus*	*Athyrium felix-femina*	*Arrhenatherum elatius*
Betula alba	*Erica arborea*	*Carduus tenuiflorus*	*Dactylis glomerata*
Castanea sativa	*Erica australis*	*Crepis vesicaria*	*Hordeum murinum*
Chamaecyparis lawsoniana	*Erica umbellata*	*Digitalis purpurea*	
Cratageus monogyna	*Genista triacanthos*	*Juncus effusus*	
Cupressus lusitanica	*Halimium umbellatum*	*Lepidophorum repandum*	
Eucalyptus globulus	*Ilex aquifolium*	*Narcissus triandrus*	
Fraxinus sp.	*Lavandula stoechas*	*Omphalodes nitida*	
Laurus nobilis	*Lithospermum diffusum*	*Plantago coronopus*	
Pinus nigra	*Pterospartum tridentatum*	*Plantago lanceolata*	
Pinus pinaster	*Rubus ulmifolius*	*Potentilla erecta*	
Pseudotsuga menziesii	*Ulex minor*	*Pteridium aquilinum*	
Quercus pyrenaica		*Sanguisorba minor*	
Quercus robur		*Taraxacum officinale*	
Salix atrocinerea		*Trifolium* sp.	
		Tuberaria lignosa	

Arboreous species		
	Blank field 400x	Phase contrast 400x
Acacia dealbata		
- Bottom page (Polygonal cells, medium density, medium size, anomocytic stomata, tector trichomes)		
- Up page (Polygonal cells, médium density, medium size, anomocytic stomata, tector trichomes)		
Acacia melanoxylon		
- Bottom page (Polygonal cells with veins, high density, small size, anomocytic stomata, tector trichomes)		
- Up page (Polygonal cells with veins, high density, small size, anomocytic stomata, tector trichomes)		

- Stem (Polygonal cells with veins, high density, small size, anomocytic stomata, tector trichomes)		
Betula alba		
- Bottom page (Polygonal rounded cells, medium density, medium size, anomocytic stomata)		
- Up page (Polygonal rounded cells, medium density, medium size, anomocytic stomata)		
- Stem (Polygonal cells, medium density, small size)		

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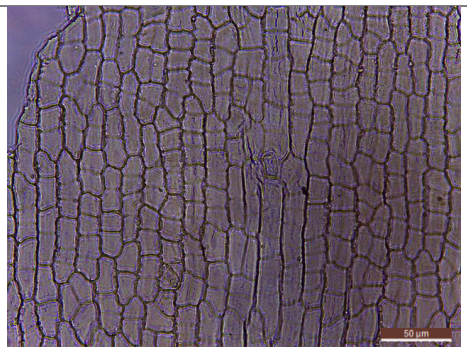
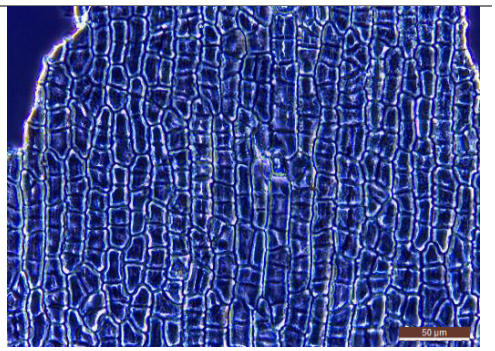
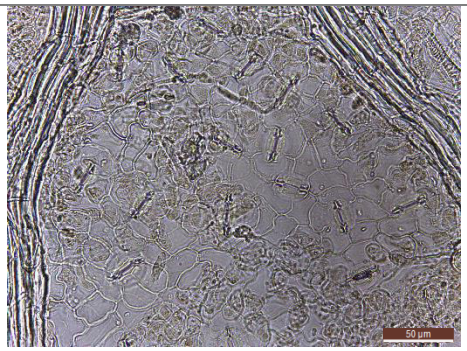
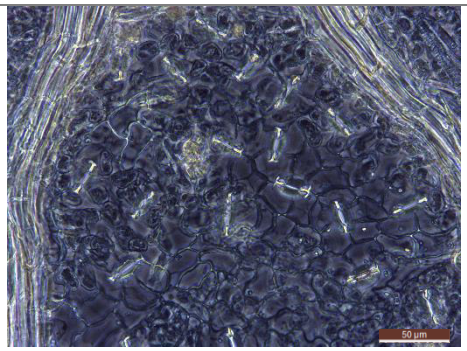
(Polygonal cells, high density, small size, tector trichomes)

Castanea sativa

- Bottom page
(Polygonal mildly cells, big density, small size, anomocytic stomata)

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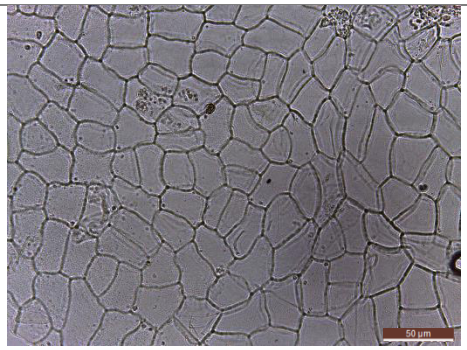
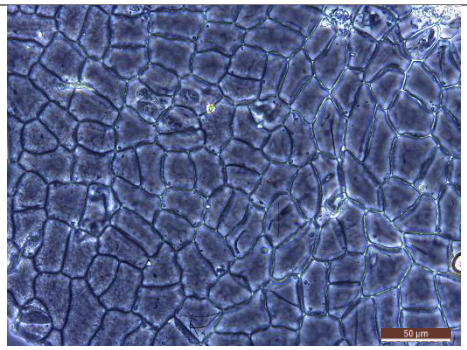
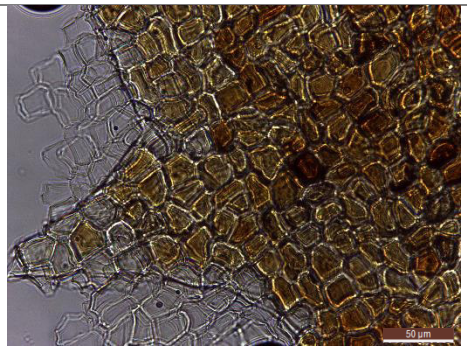
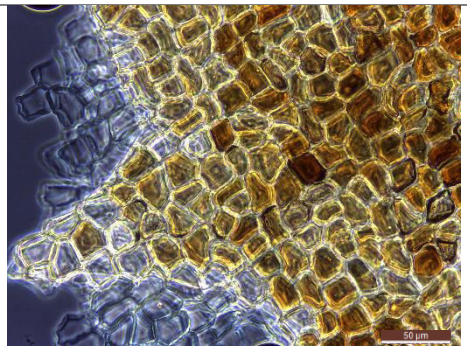
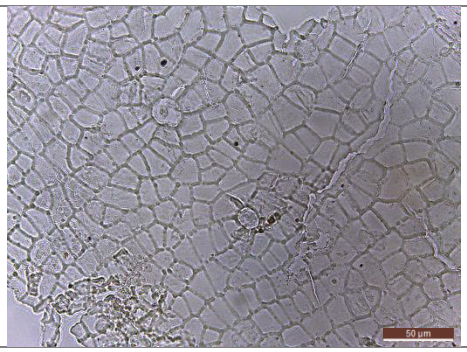
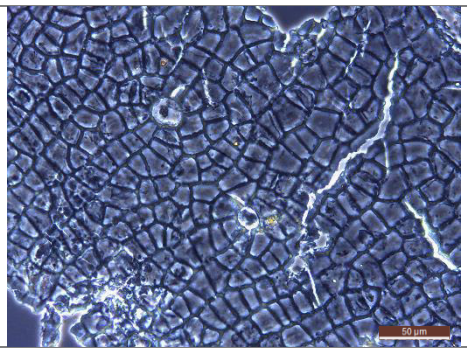
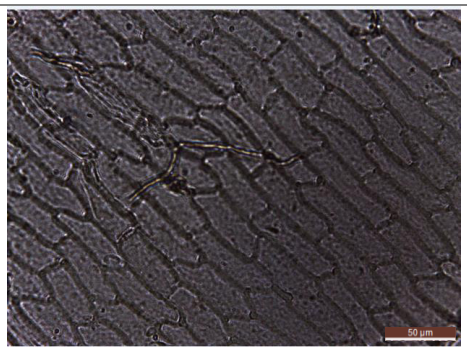
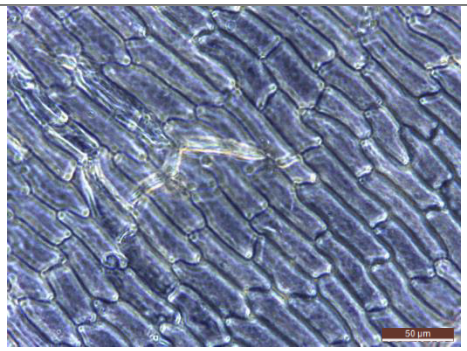
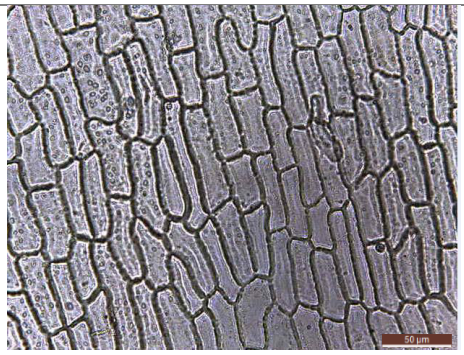
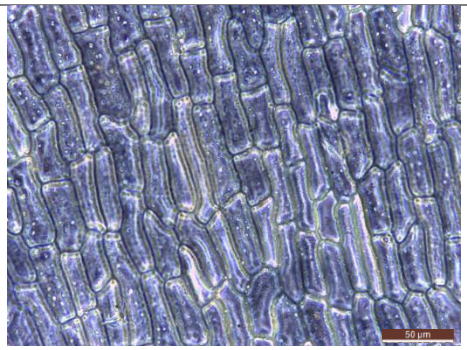
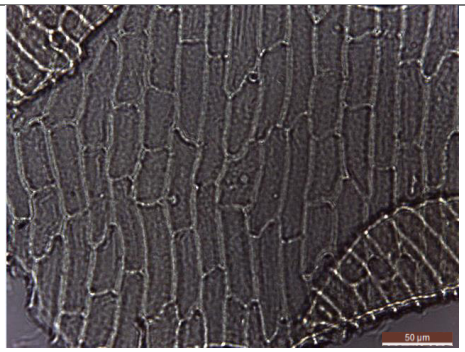
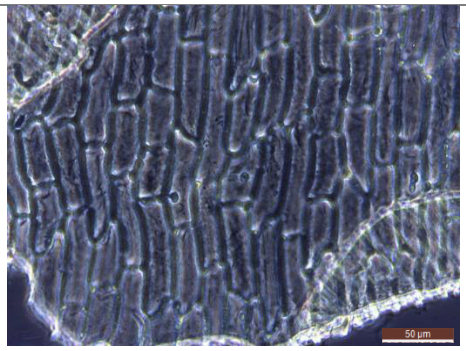



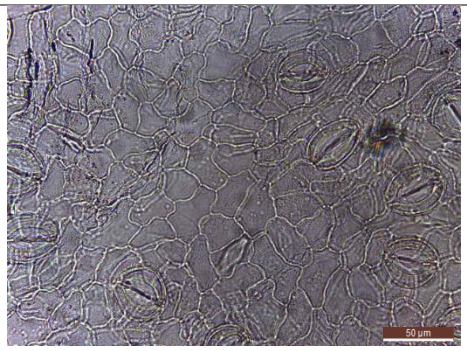
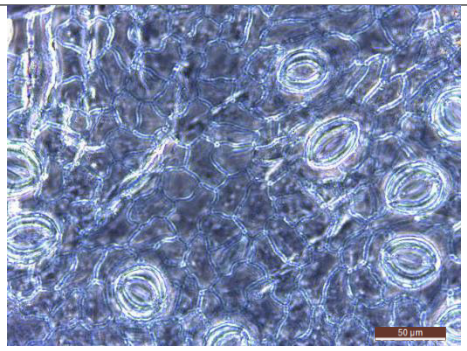
- Stem
(Polygonal cells, high density, small size)

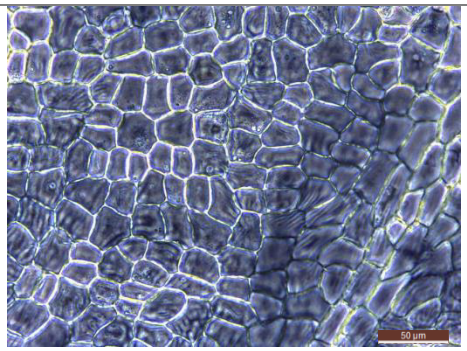
- Sprout (Polygonal cells, high density, small size)		
Chamaecyparis lawsoniana		
- **Bottom page** (Rectangular cells, medium density, medium size)		
- Up page (Rectangular cells, medium density, medium size)		
- Stem (Rectangular cells, medium density, medium size)		

Crataegus monogyna

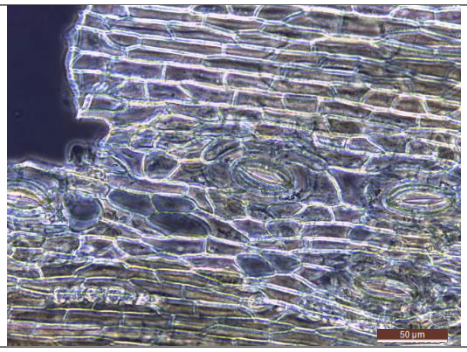
- Bottom page
(Polygonal largely cells, medium density, médium size, anomocytic stomata)

- Up page
(Polygonal largely cells, medium density, medium size)

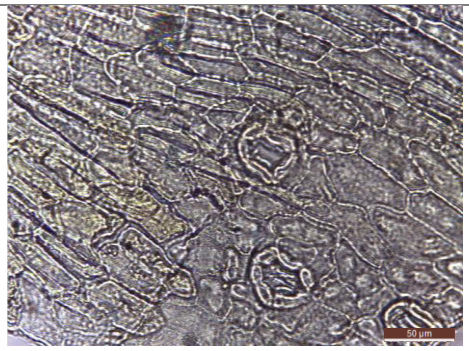
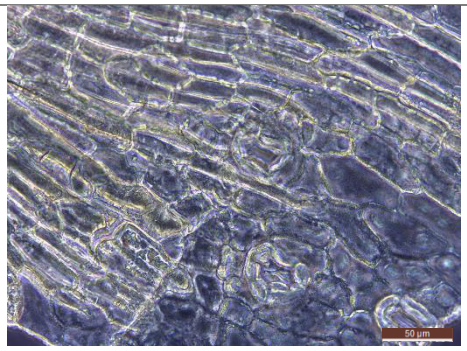



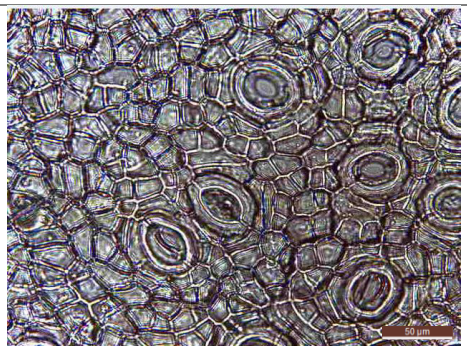
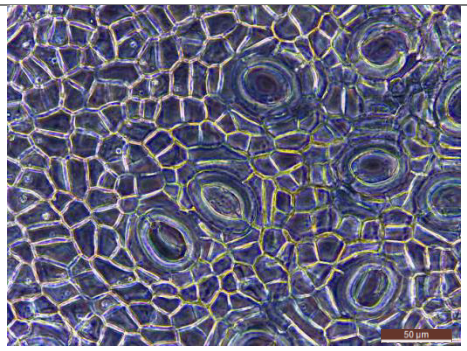
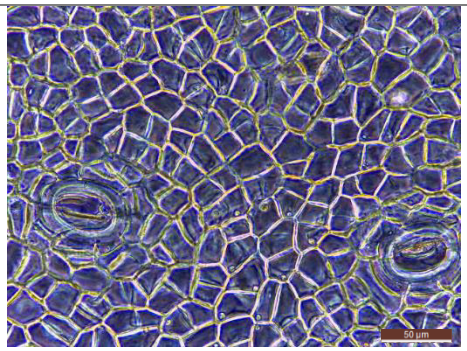
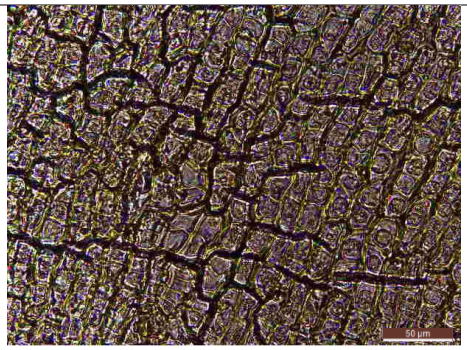
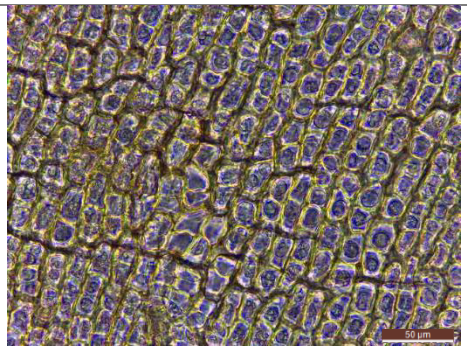
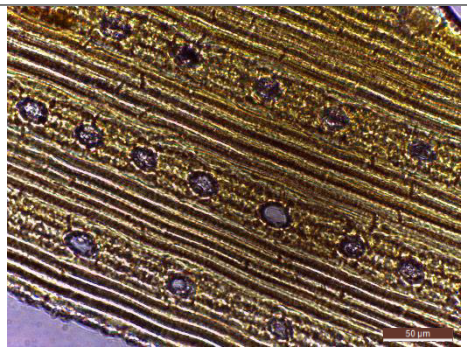
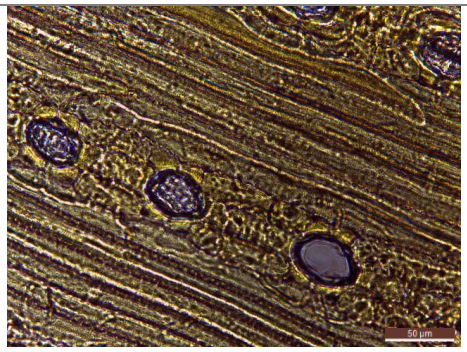
- Stem
(Rectangular cells, low density, big size, anomocytic stomata)

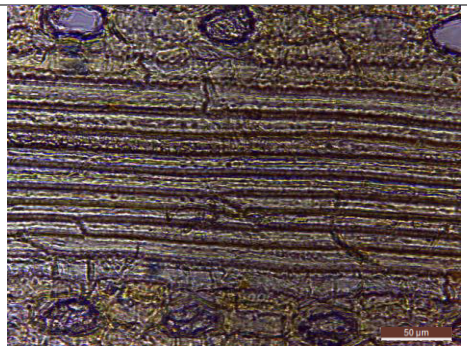
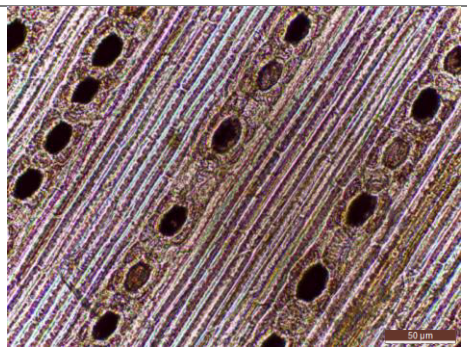
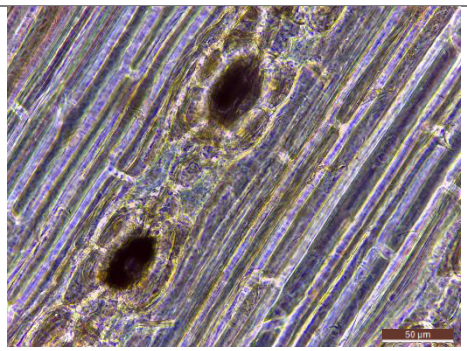
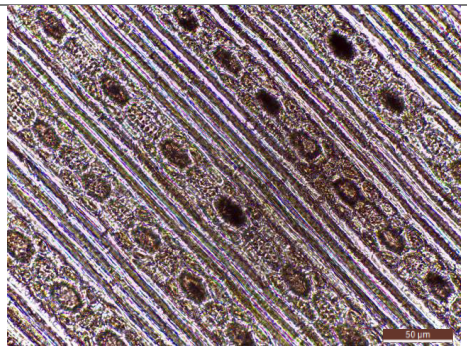
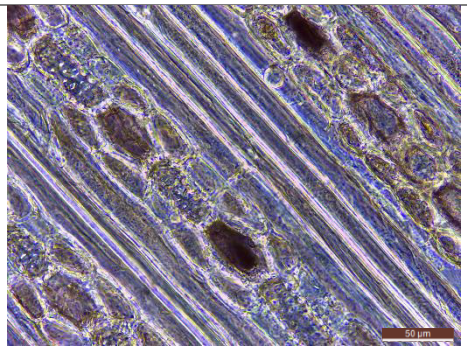
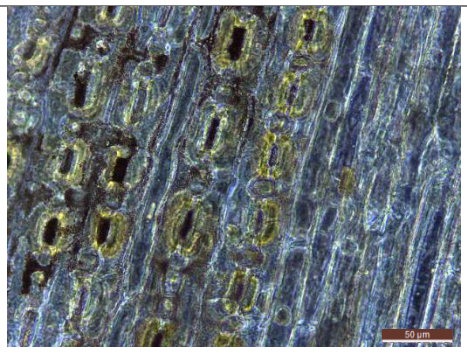



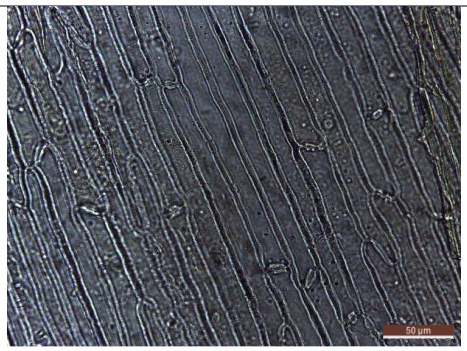
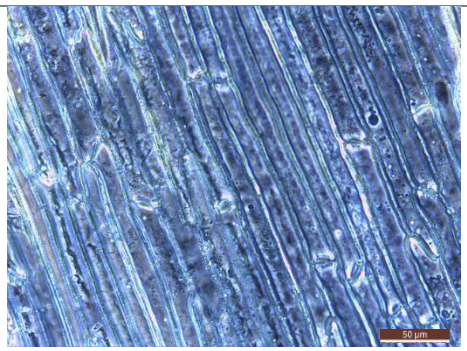
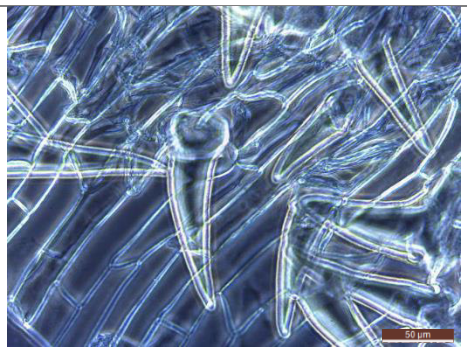
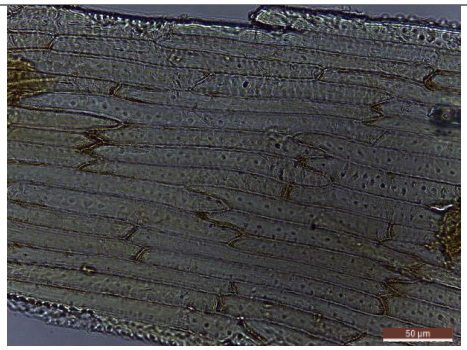
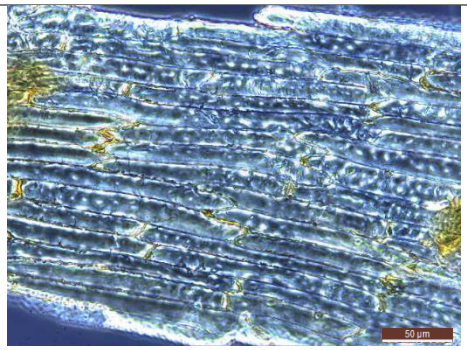
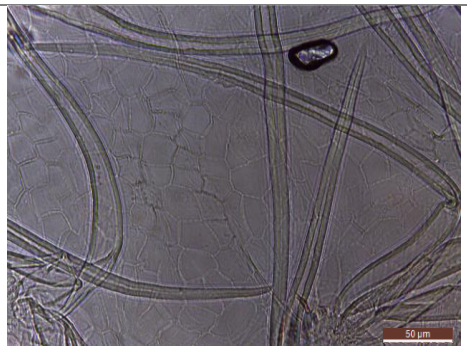
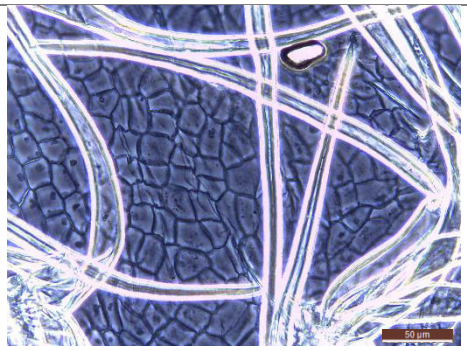
Cupressus lusitânica

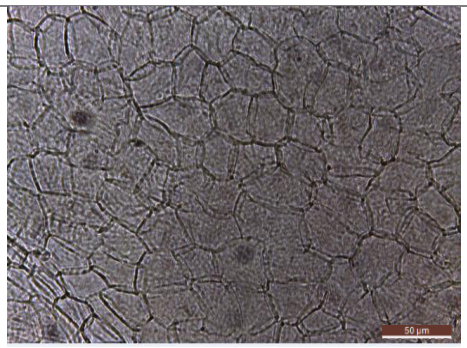
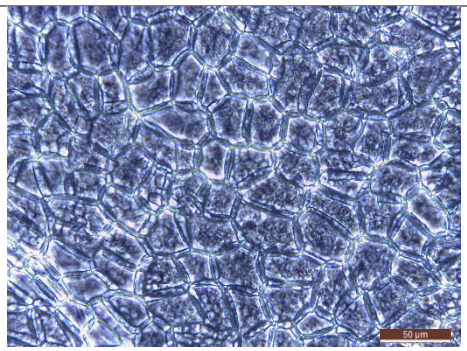
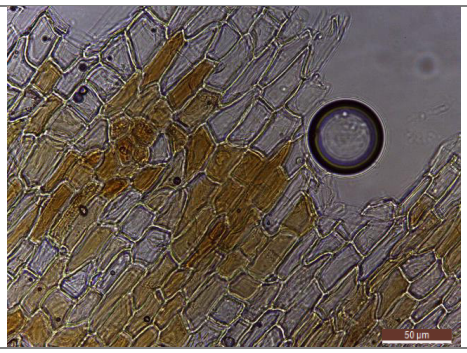
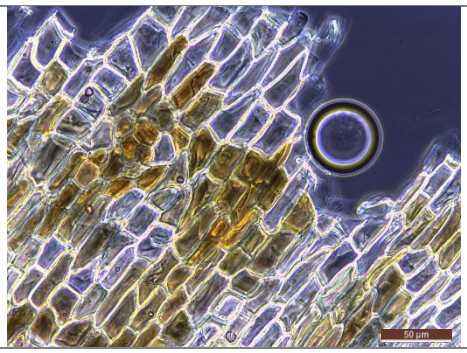
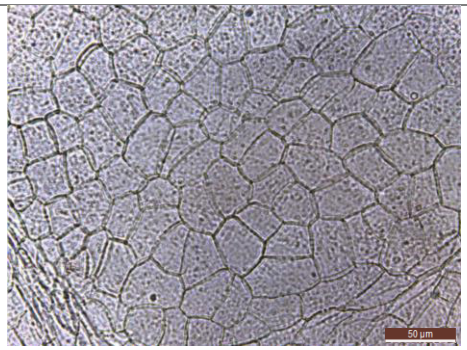
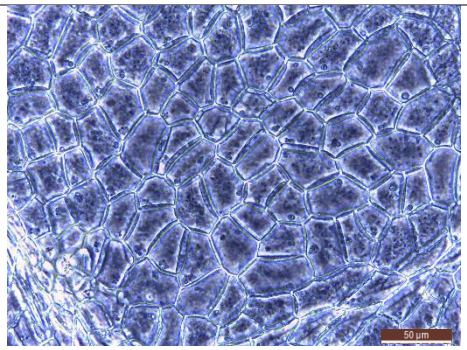
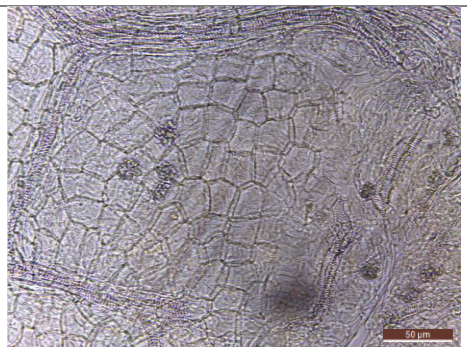
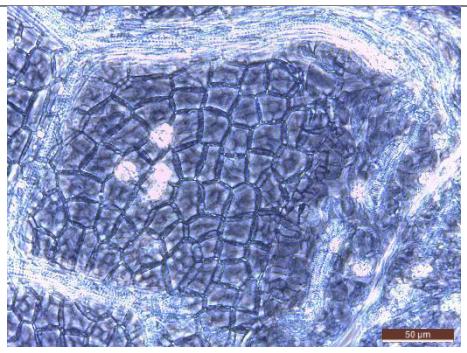
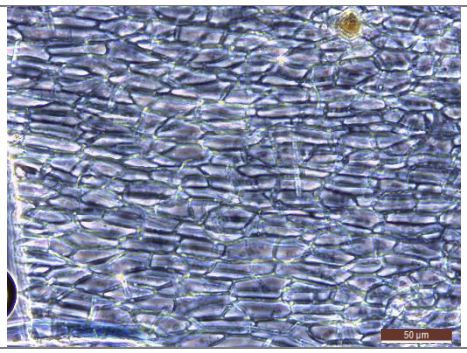
- Up page
(Polygonal cells, low density, large size, anomocytic stomata)

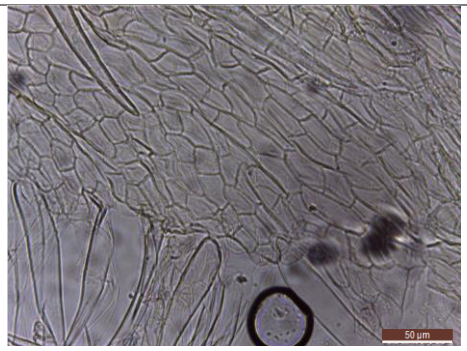
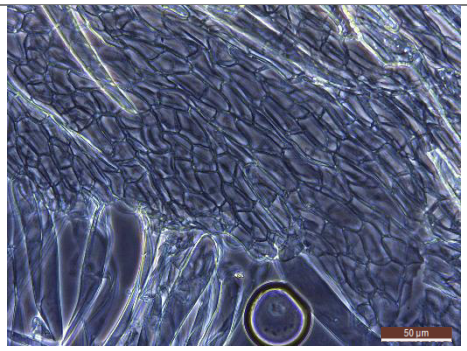
Eucalyptus globulus		
- Bottom page (Polygonal cells, medium density, medium size, paracytic stomata)		
- Up page (Polygonal cells, medium density, medium size, paracytic stomata)		
- Stem (Polygonal cells, medium density, medium size)		
Pinus nigra		
- Bottom page (Rectangular cells, low density, big size, stomata in line)		

- Up page (Rectangular cells, low density, big size, stomata in line)		
Pinus pinaster		
- Bottom page (Rectangular cells, low density, big size, stomata in line)		
- Up page (Rectangular cells, low density, big size, stomata in line)		
Pseudotsuga menziesii		
- Bottom page (Rectangular cells, low density, big size, stomata in queue)		

- Up page (Rectangular cells, low density, big size)		
- Stem (Rectangular cells. Low density, big size, short trichomes)		
- Sprout (Rectangular cells, low density, big size)		
Quercus Pyrenaica		
- Bottom page (Polygonal cells, high density, small size, long tector trichomes)		

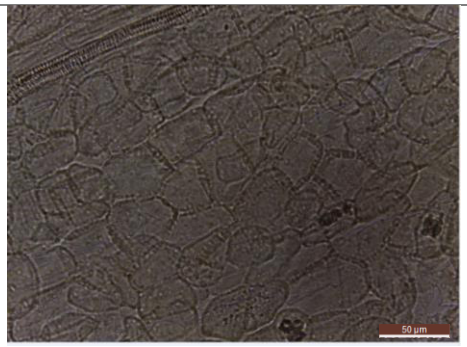
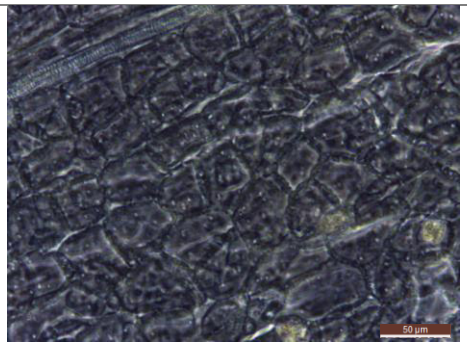
- Up page (Polygonal cells, medium density, medium size, tector trichomes)		
- Stem (Polygonal cells, medium density, medium size)		
Quercus róbur		
- Bottom page (Polygonal cells, medium density, medium size, tector trichomes)		
- Up page (Polygonal cells, medium density, medium size, tector trichomes)		
- Stem (Polygonal cells, high density, small size, low density of tector trichomes)		

- Sprout
(Polygonal cells, high density, small size, high density of tector trichomes)

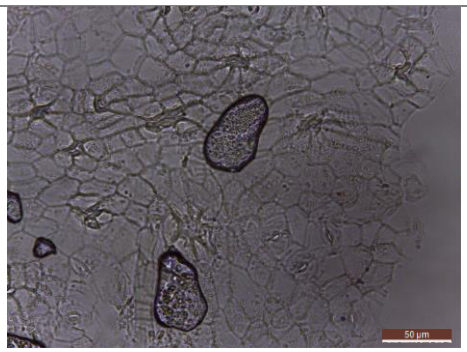
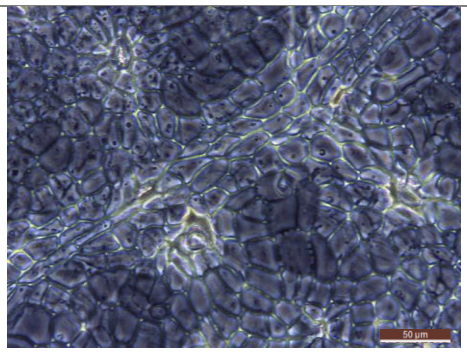



Salix atrocinerea

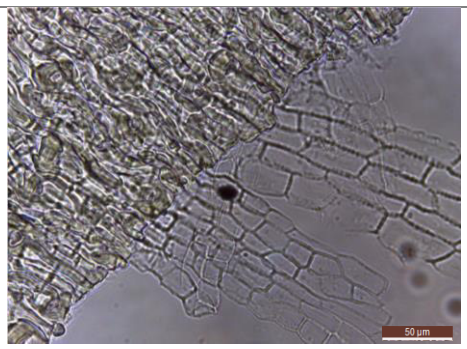
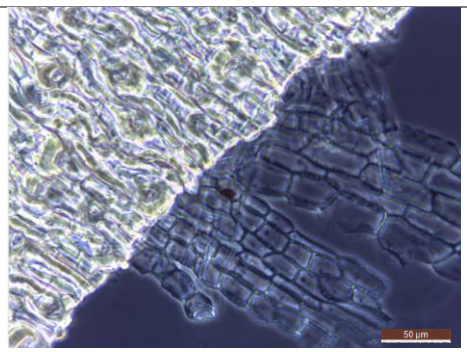
- Bottom page
(Polygonal cells, medium density, medium size, tector trichomes)

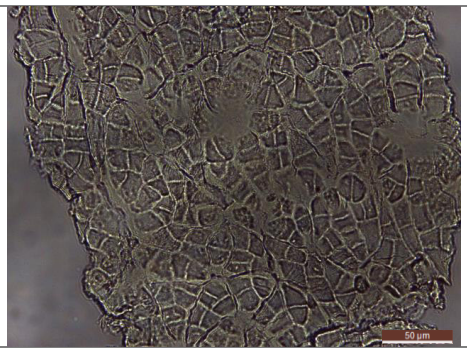
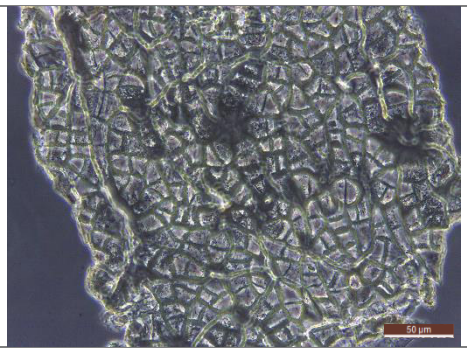



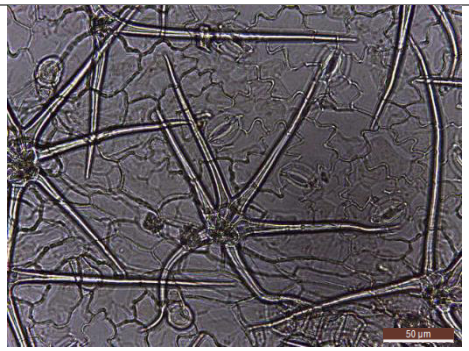
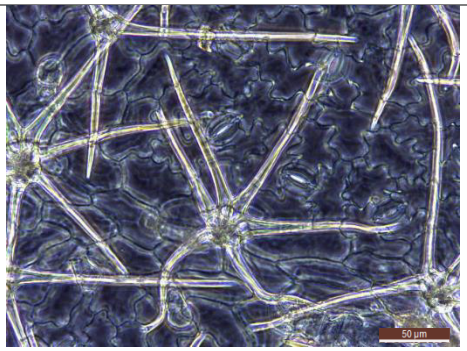
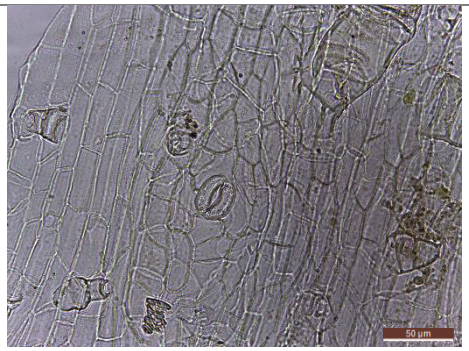
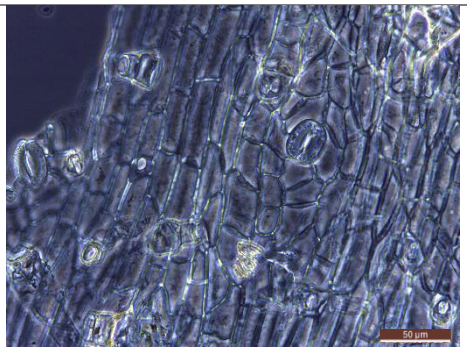
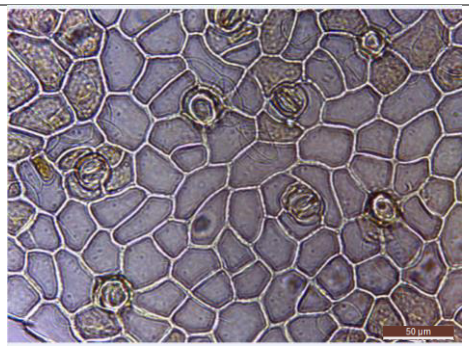
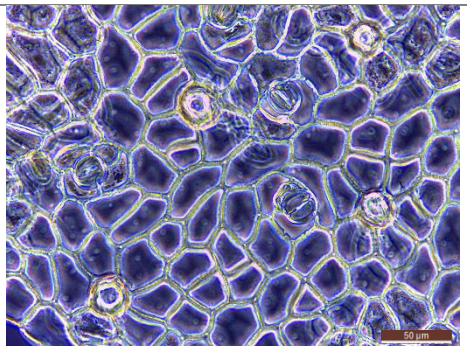
- Up page
(Polygonal cells, medium density, medium size, tector trichomes)

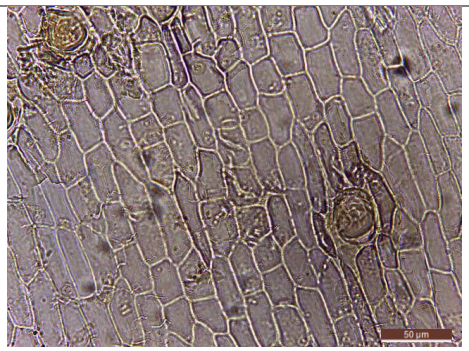
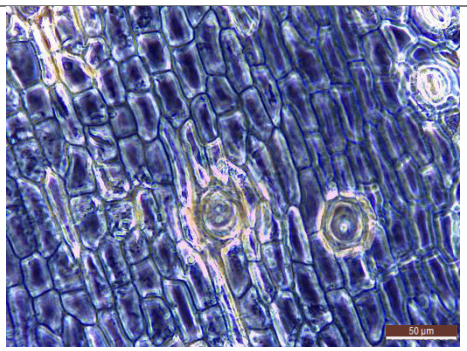
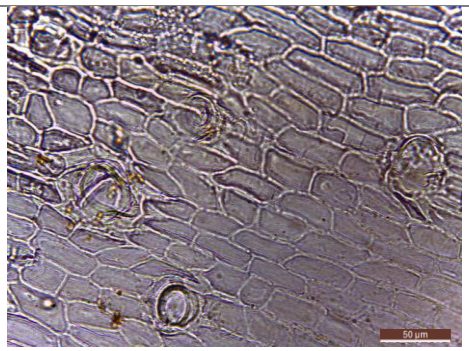
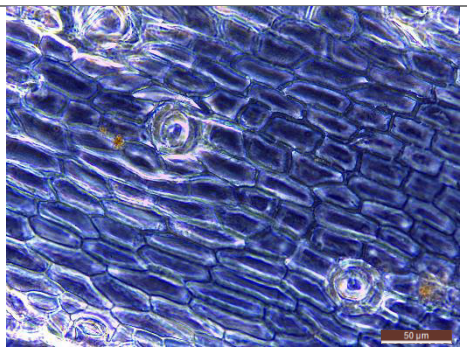
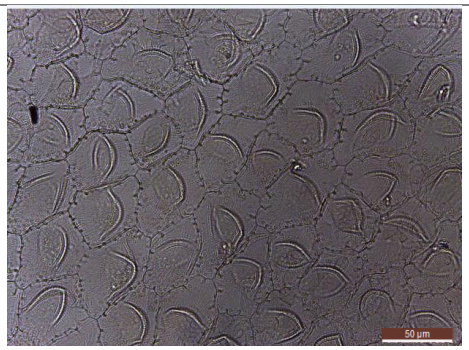
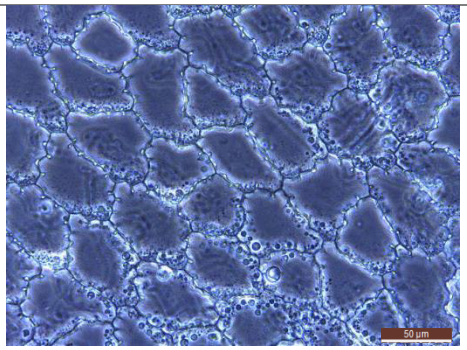
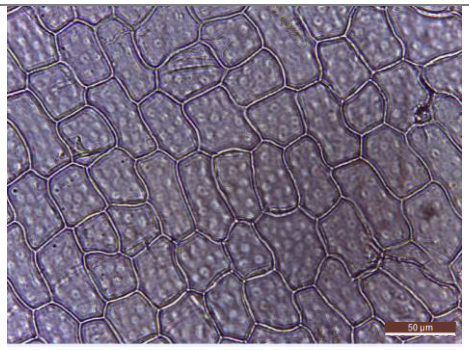
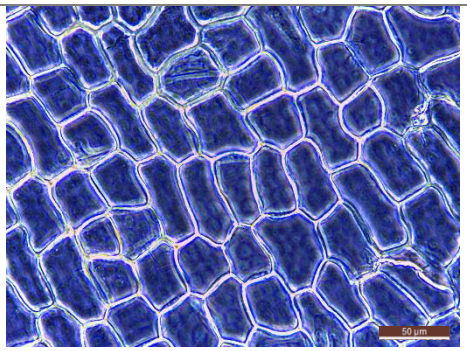



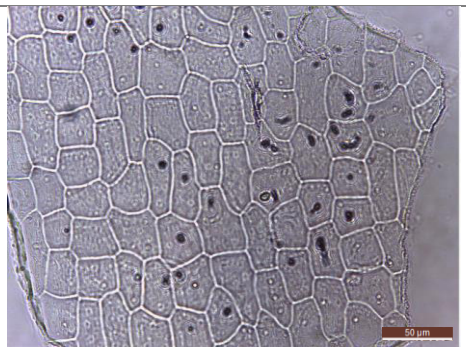
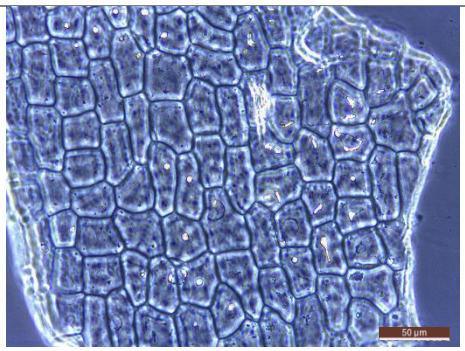
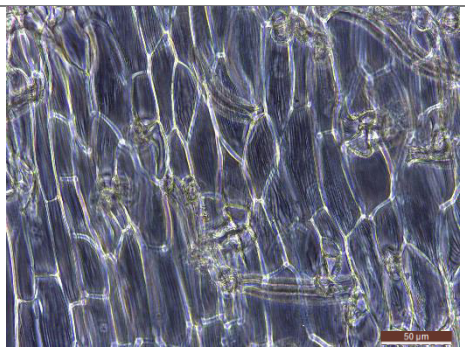
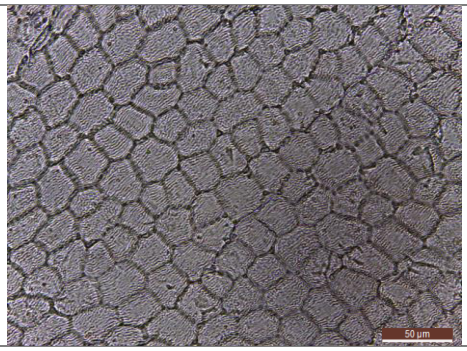
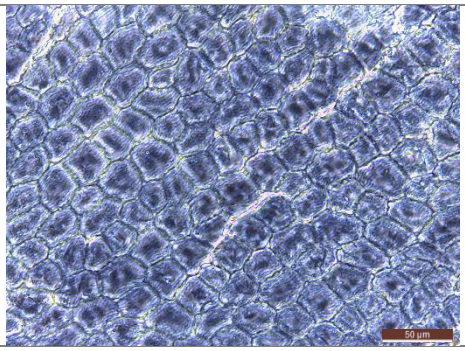
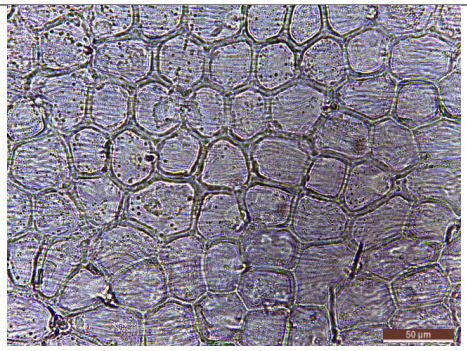
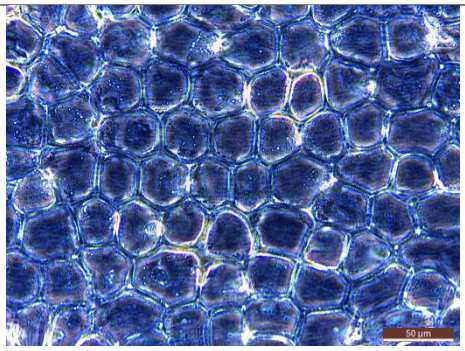
- Stem
(Polygonal cells, medium density, medium size, tector trichomes)

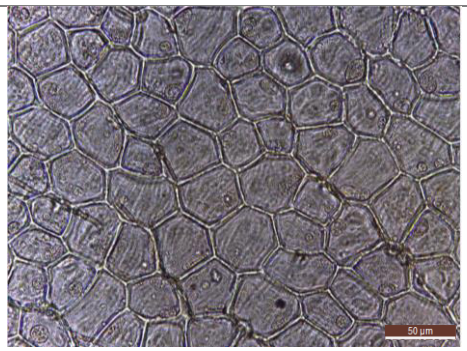
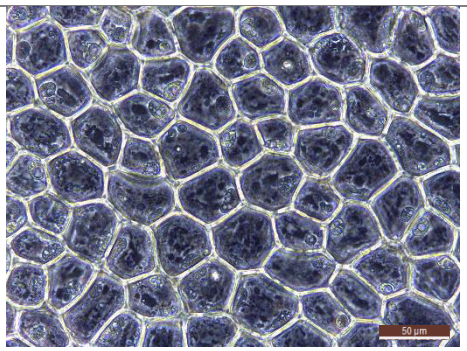
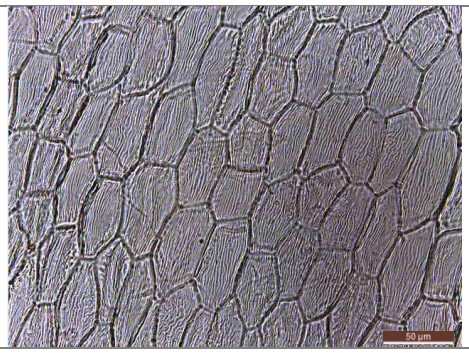
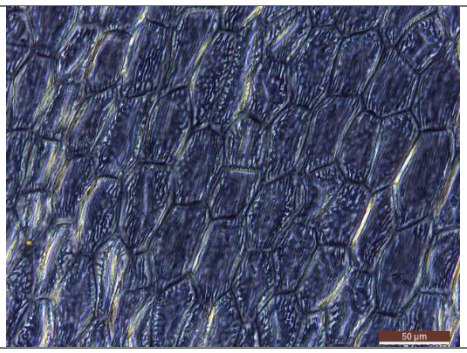
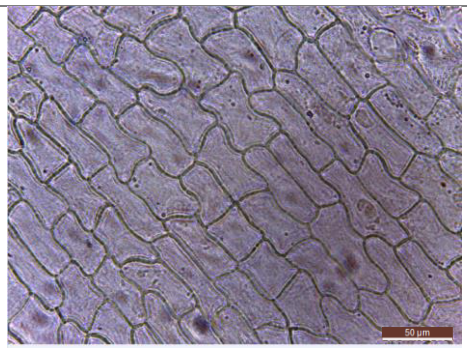
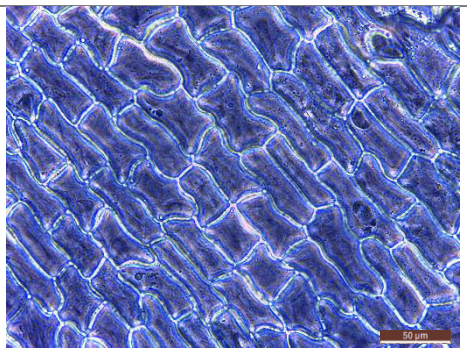
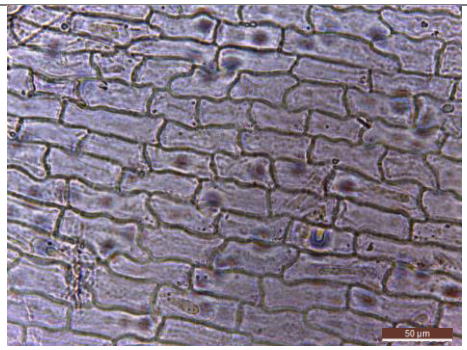
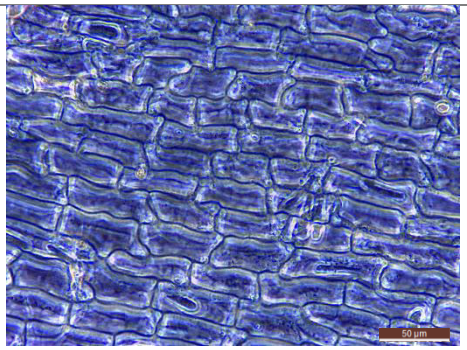



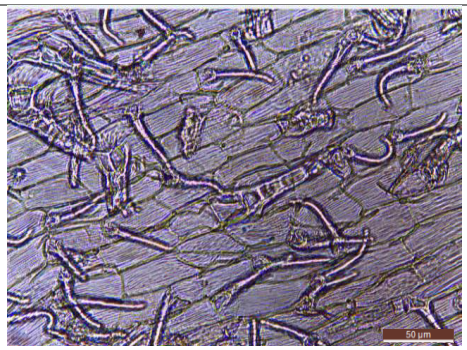
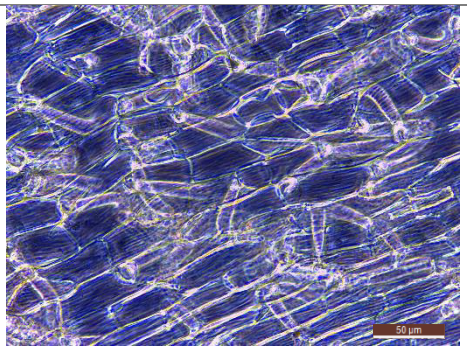
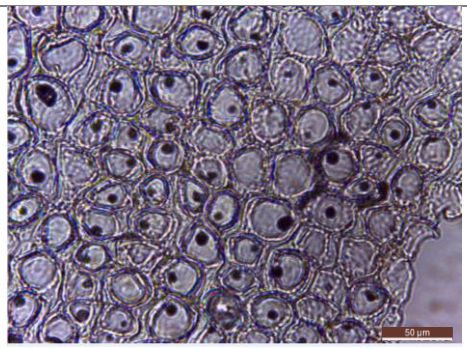
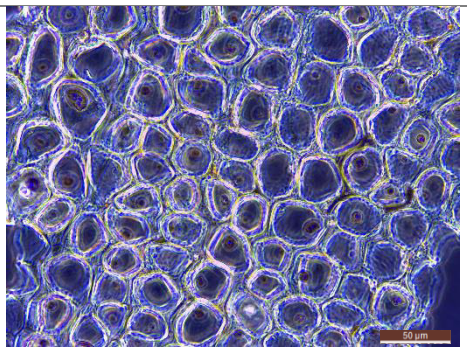
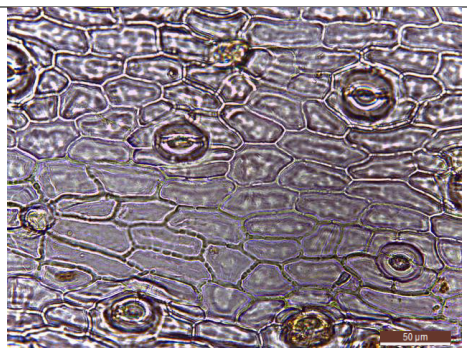
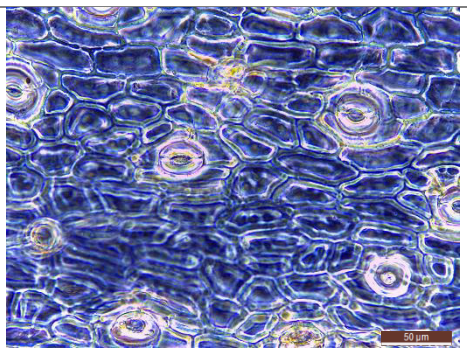
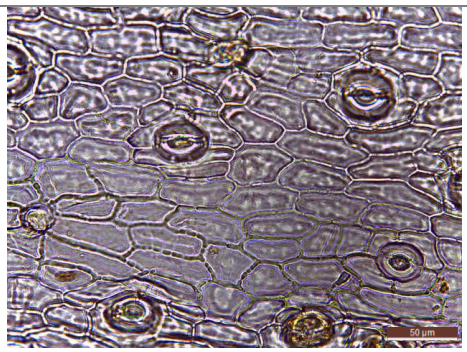
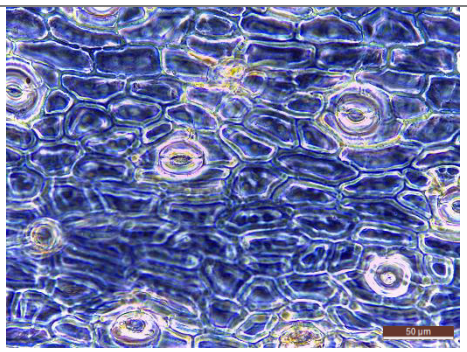
- Sprout (Polygonal cells, medium density, medium size, tector trichomes)		

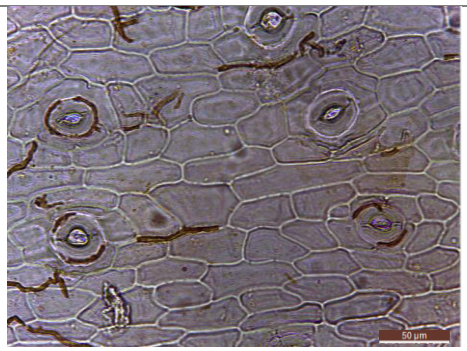
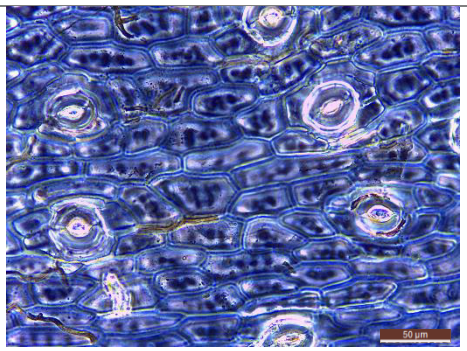
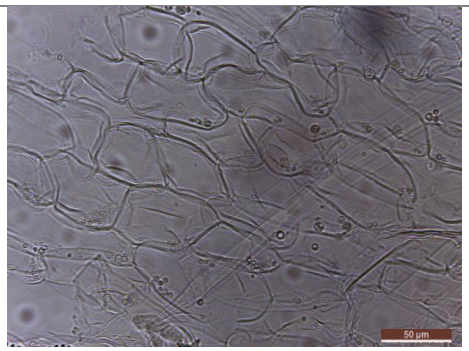
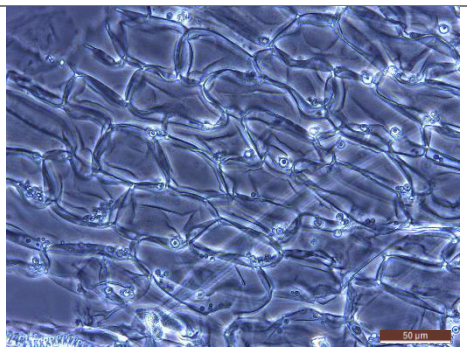
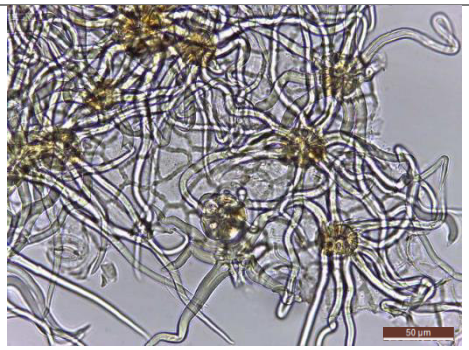
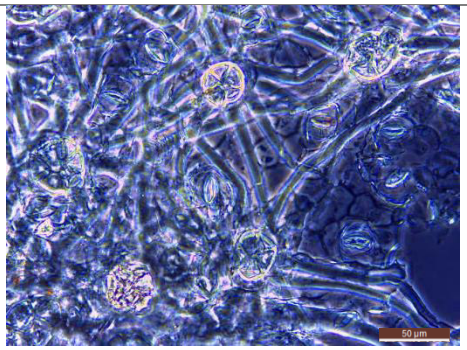
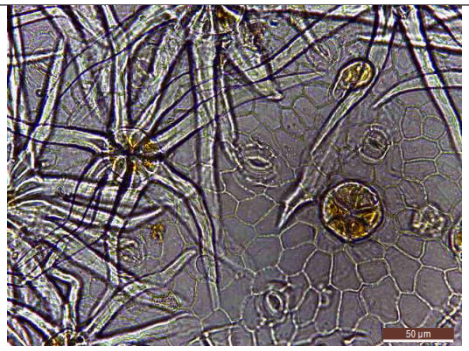
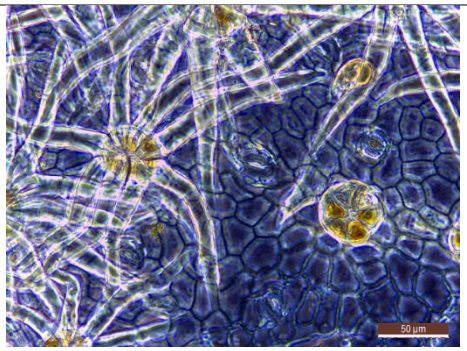
Shrub species		
Cistus monspeliensis		
- Bottom page (Puzzle cells, high density, big size, anaomocytic stomata, starry trichomes)		
- Up page (Polygonal cells, medium density, medium size, anomocytic stomata, starry trichomes)		
- Stem (Rectangular cells, high density, small size, anomocytic stomata randomly distributed, tector trichomes)		
Cytisus striatus		
- Bottom page (Polygonal cells, low density, big size, anomocytic, ciclocitic and tetracyclic stomata, tector trichomes)		

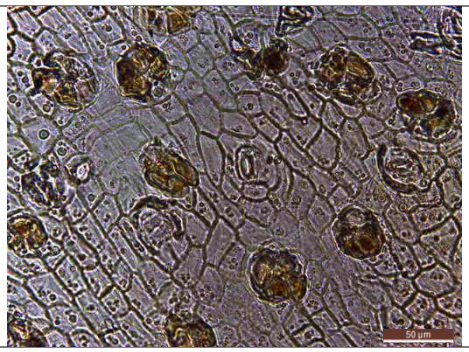
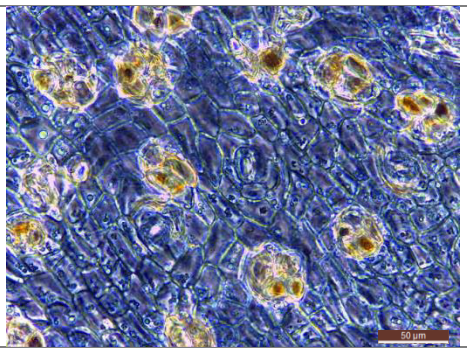
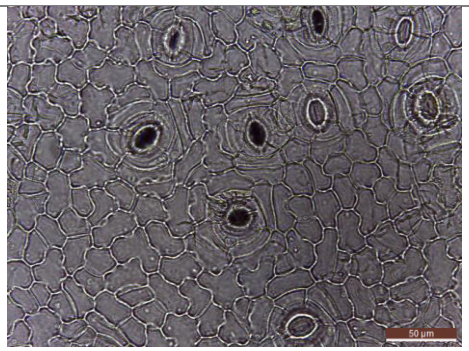
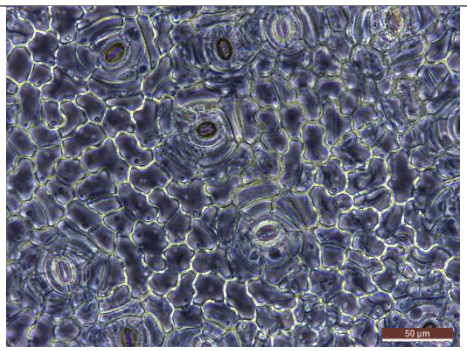
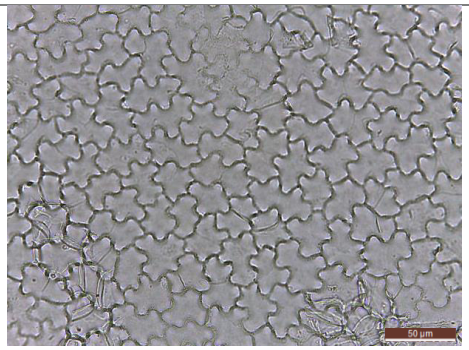
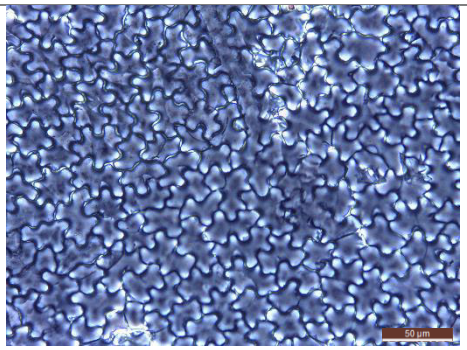
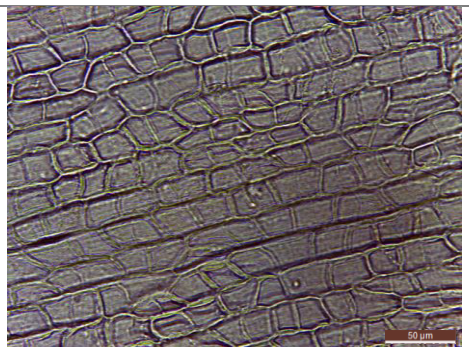
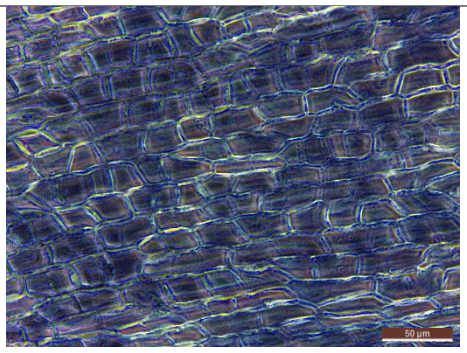
- Up page (Rectangular cells, low density, small size, anomocytic and ciclocytic stomata, tector trichomes)		
Stem (Rectangular cells, low density, small size, anomocytic and ciclocytic stomata, tector trichomes)		
- Flower (Polygonal cells, medium density, medium size)		
Erica arborea		
- Bottom page (Polygonal cells, low density, big size, tector trichomes)		

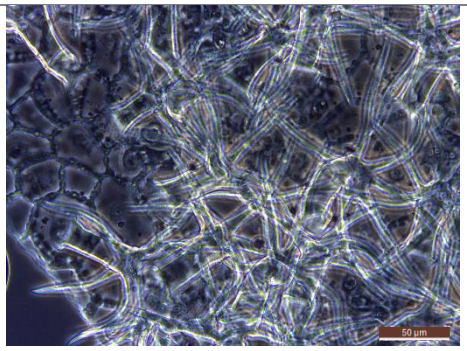
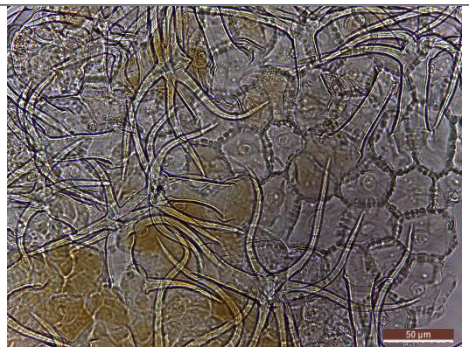
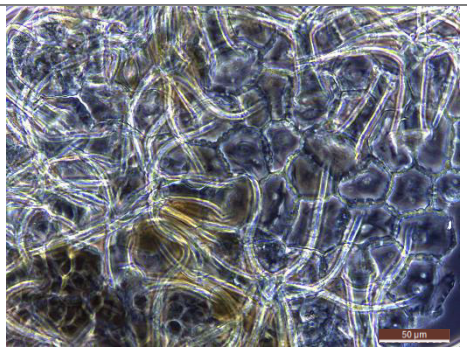
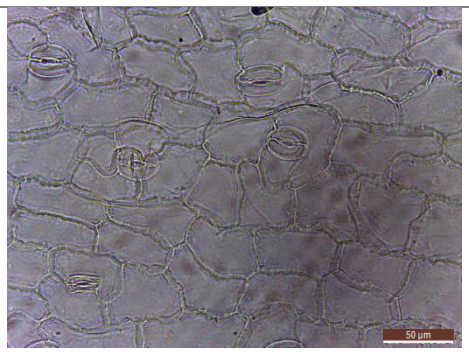
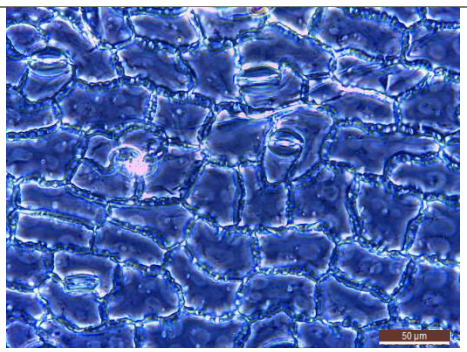
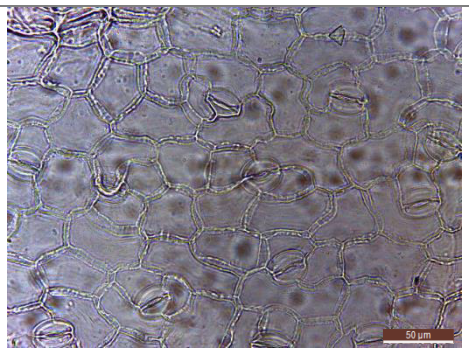
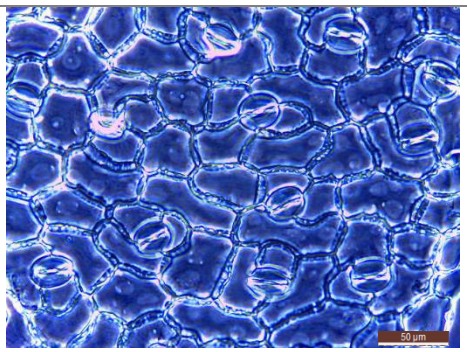
- Up page (Polygonal cells, low density, big size, tector trichomes)		
- Stem (Rectangular cells, medium density, medium size, tector trichomes)		
- Flower (Polygonal pentagonal cells, high density, small size)		
Erica australis		
- Bottom page (Polygonal cells, medium density, medium size, tector trichomes)		

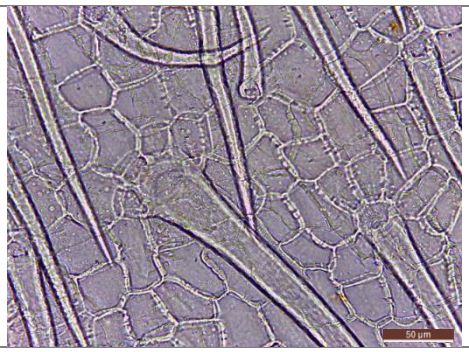
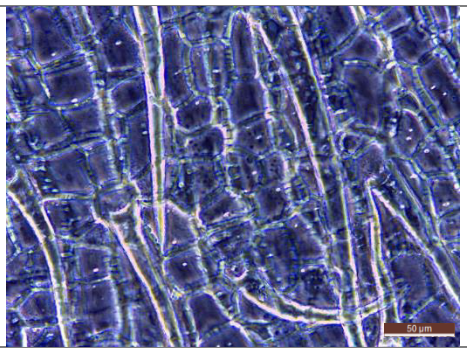
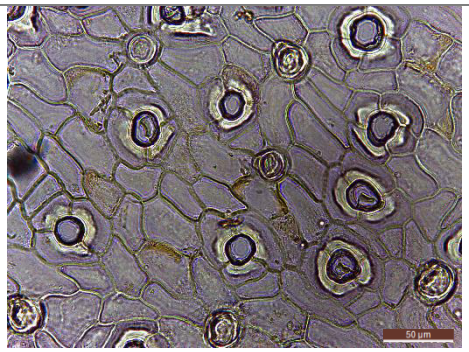
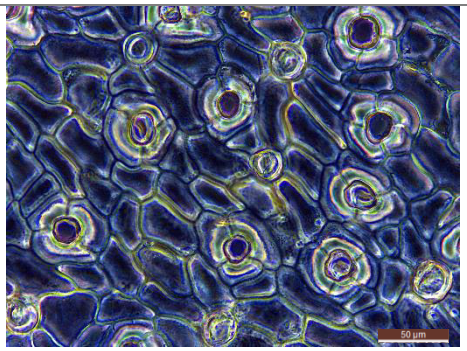
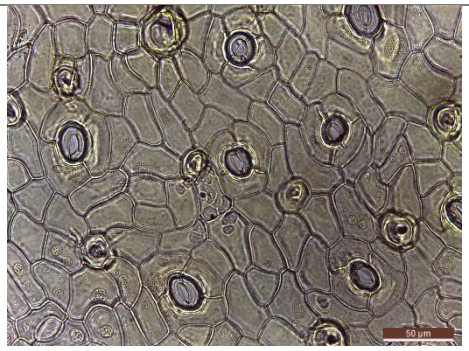
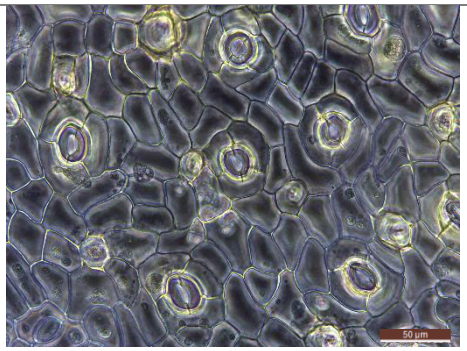
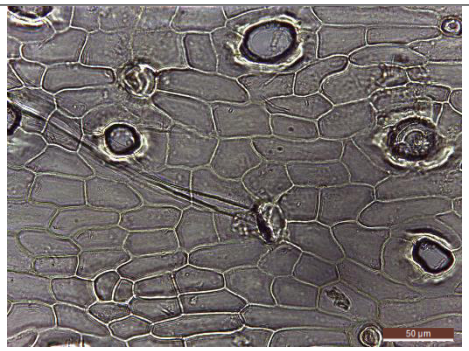
- Up page (Polygonal cells, medium density, medium size, tector trichomes)		
- Flower (Polygonal cells, low density, big size)		
Erica umbellata		
-Bottom page (Rectangular cells, medium density, medium size, tector trichomes)		
- Up page (Rectangular cells, medium density, medium size, tector trichomes)		

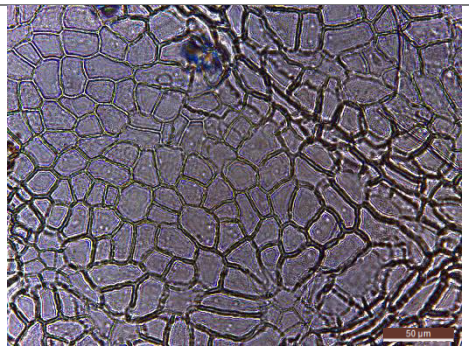
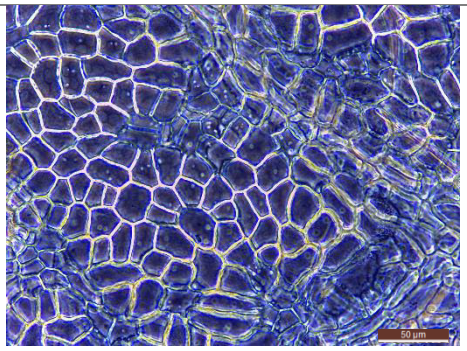
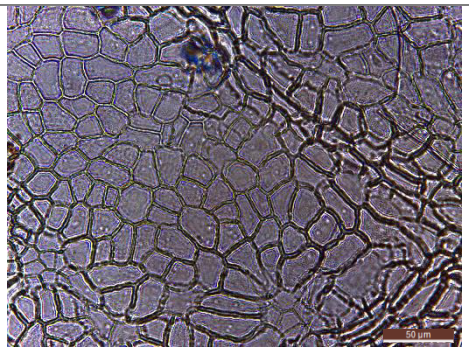
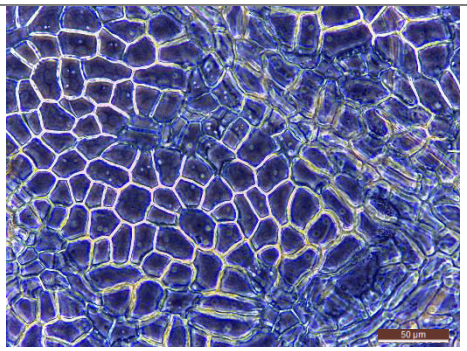
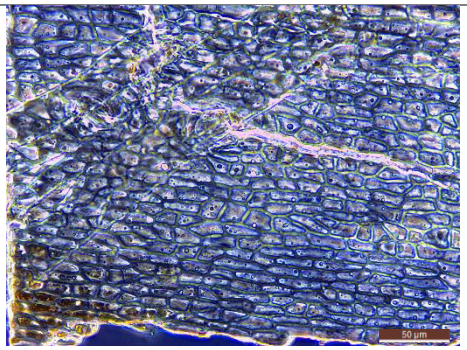
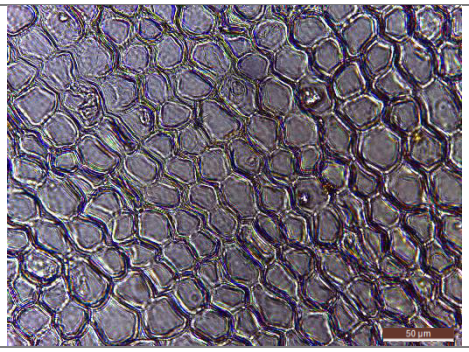
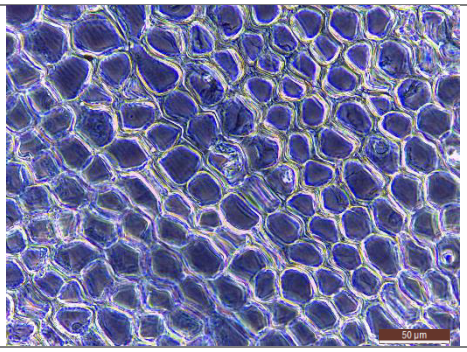
- Stem (Rectangular cells, medium density, medium size, tector trichomes)		
- Flower (Polygonal cells, medium density, medium size)		
Genista triacanthos		
- Bottom page (Polygonal cells, medium density, medium size, anisocytic, tetracyclic and ciclocytic stomata, tector trichomes)		
- Up page (Polygonal cells, medium density, medium size, anisocytic, tetracyclic and ciclocytic stomata, tector trichomes)		

- Stem (Polygonal cells, medium density, medium size, anisocytic, tetracyclic and ciclocytic stomata, tector trichomes)		
- Flower (Polygonal cells, low density, big size)		
Halimium umbellatum		
- Bottom page (Polygonal cells, medium density, medium size, anomocytic stomata, starry trichomes)		
- Up page (Polygonal cells, medium density, medium size, anomocytic stomata, starry trichomes)		

- Stem (Polygonal cells, high density, small size, anomocytic stomata, starry trichomes)		
Ilex aquifolium		
- Bottom page (Puzzle cells, high density, big size, ciclocytic stomata)		
- Up page (Puzzle cells, high density, low size)		
- Stem (Polygonal cells, high density, small size)		

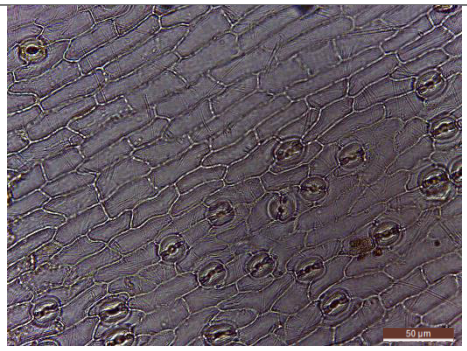
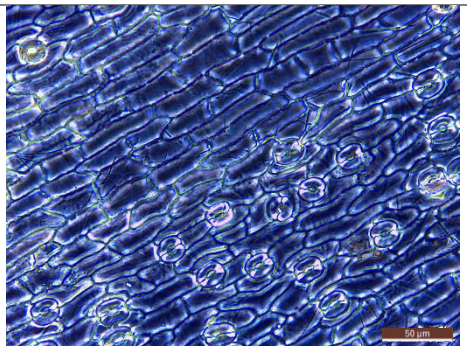
Lavandula stoechas		
- Bottom page (Polygonal cells, medium density, medium size, starry trichomes)		
- Up page (Polygonal cells, medium density, medium size, starry trichomes)		
Lithospermum diffusum		
- Bottom page (Polygonal largely cells, low density, big size, anomocytic stomata, tector trichomes)		
- Up page (Polygonal largely cells, low density, big size, anomocytic stomata, tector trichomes)		

- Stem (Polygonal cells, medium density, medium size, short tector trichomes)		
Pterospartum tridentatum		
- Bottom page (Polygonal cells, medium density, medium size, anomocytic, cicliocytic and tetracyclic stomata, tector trichomes)		
- Up page (Polygonal cells, medium density, medium size, anomocytic, cicliocytic and tetracyclic stomata, tector trichomes)		
- Stem (Polygonal cells, medium density, medium size, anomocytic, cicliocytic and tetracyclic stomata, tector trichomes)		

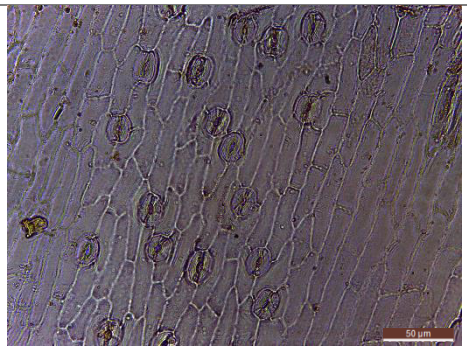
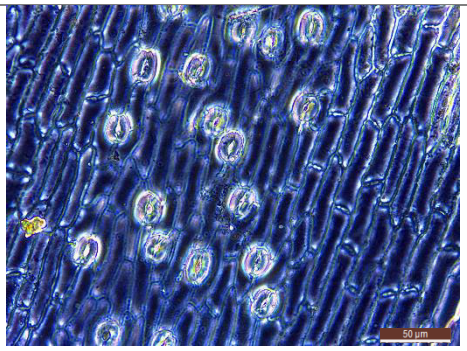
Rubus ulmifolius		
- Bottom page (Polygonal cells in rosettes, medium density, medium size)		
- Up page (Polygonal cells in rosettes, medium density, medium size)		
- Stem (Polygonal cells, high density, small size)		
- Flower (Polygonal cells, medium density, medium size)		

Ulex minor

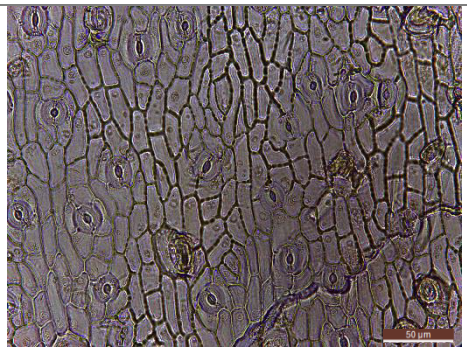
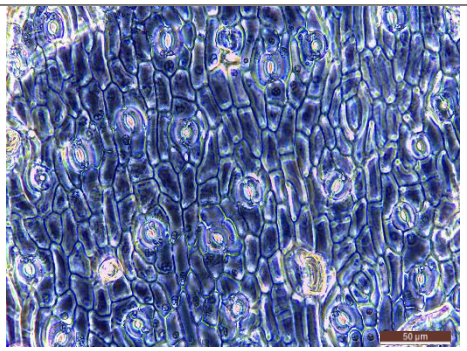
- Bottom page
(Rectangular cells, medium density, medium size, anomocytic stomata)

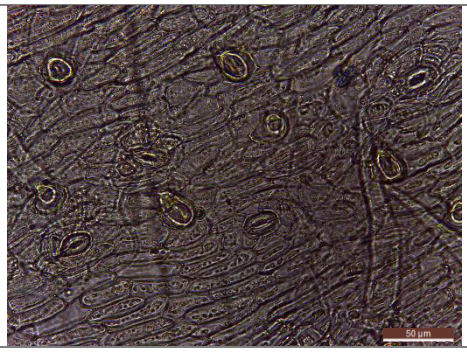
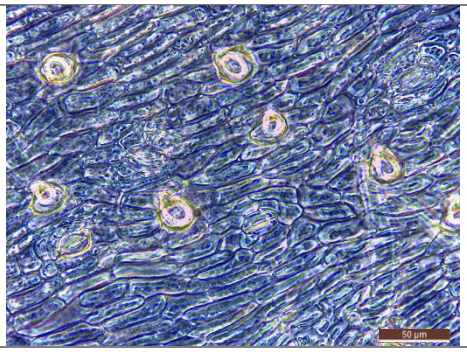
- Up page
(Rectangular cells, medium density, medium size, anomocytic stomata)

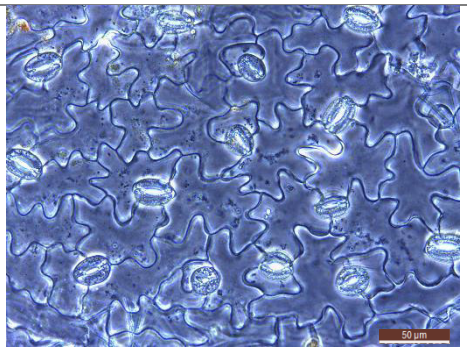
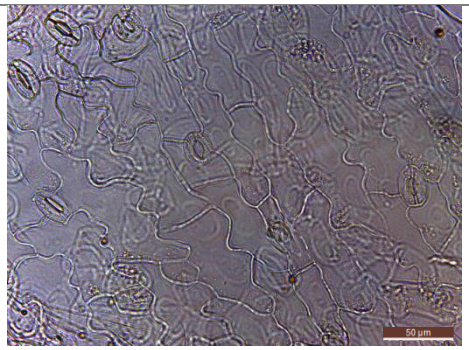
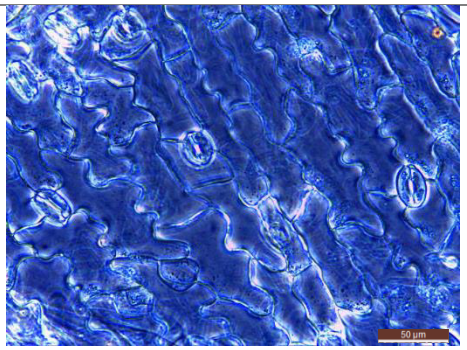
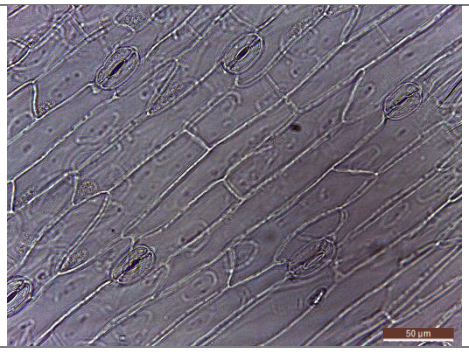
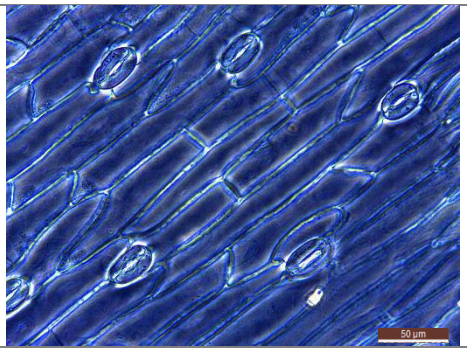
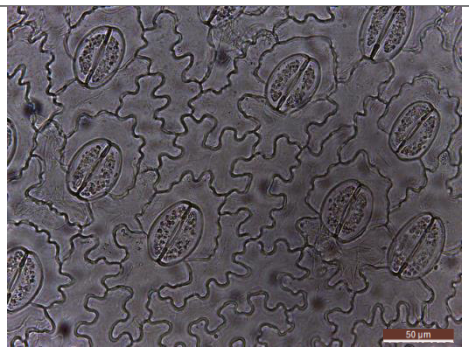
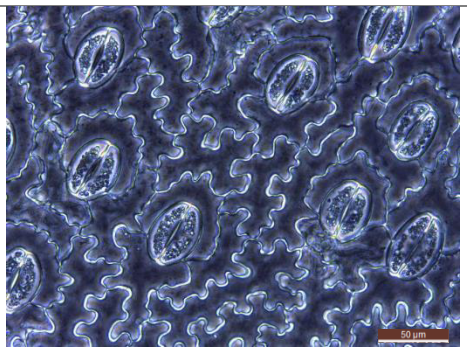



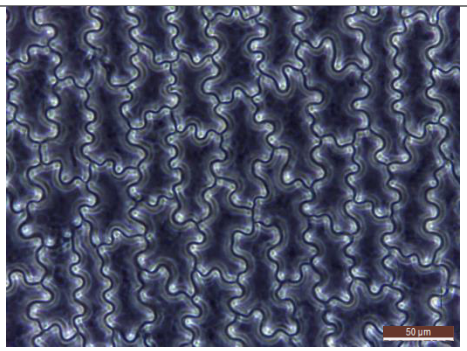
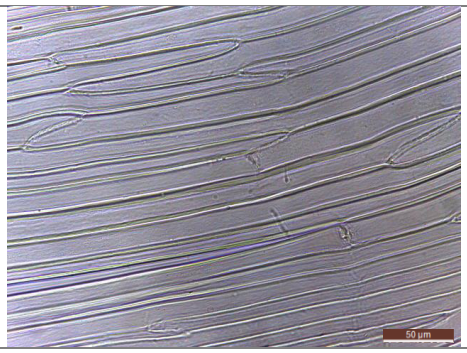
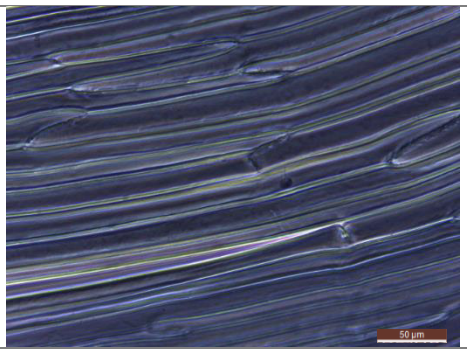
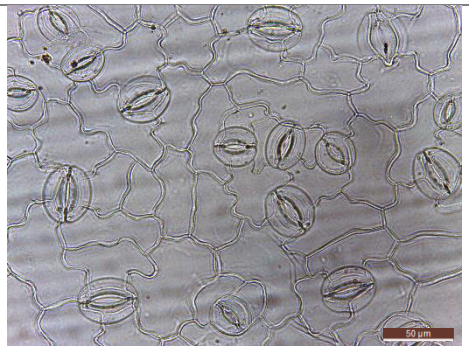
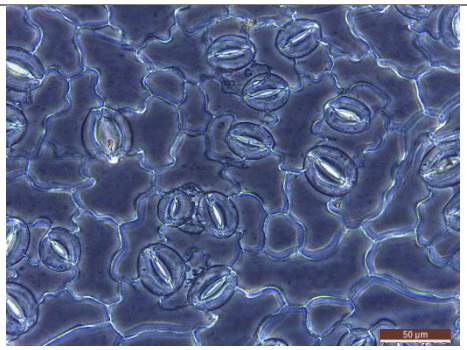
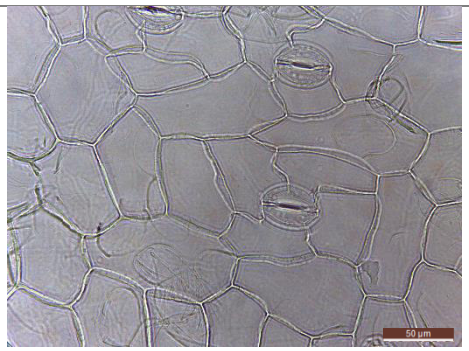
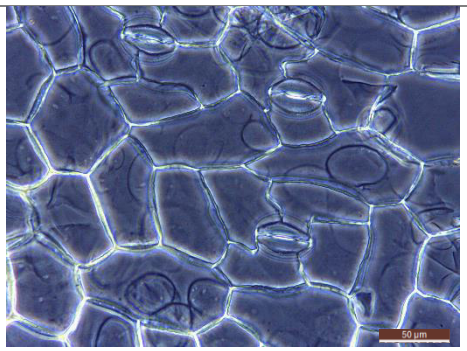
- Stem
(Rectangular cells, medium density, medium size, anomocytic stomata, tector trichomes)

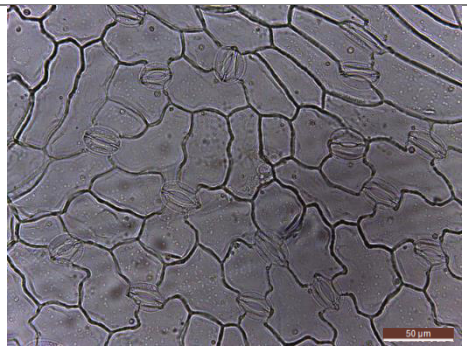
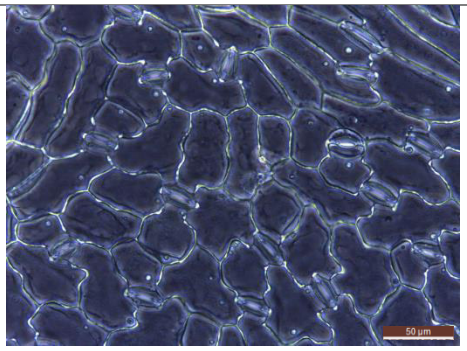
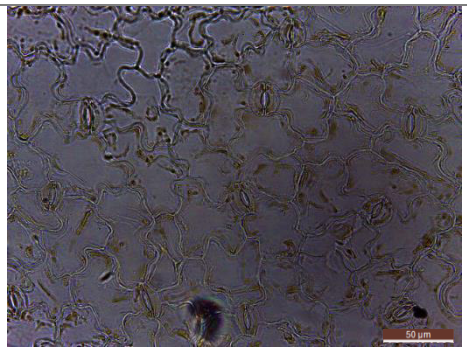
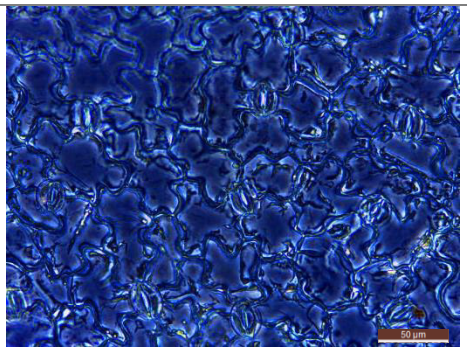
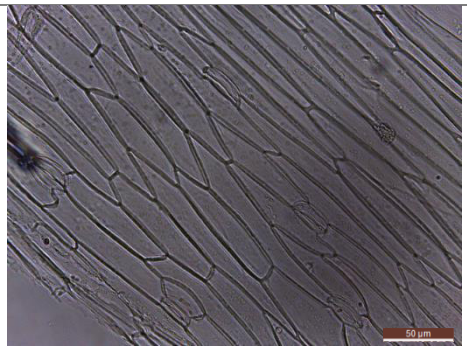
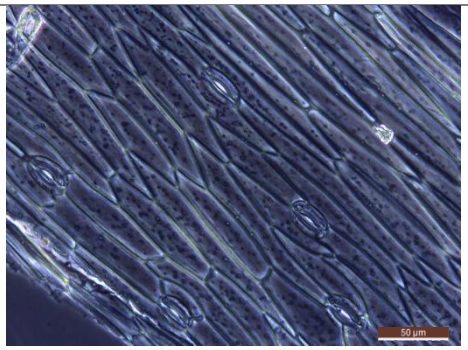
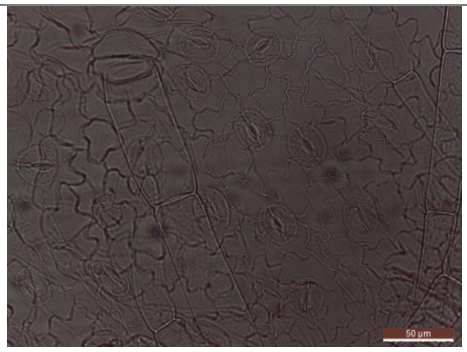
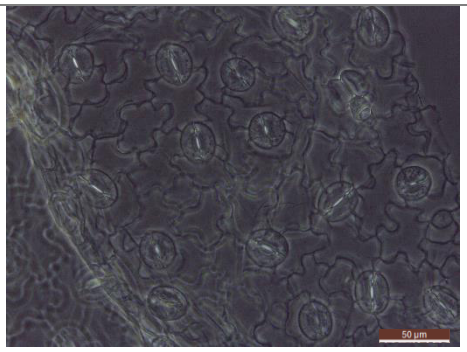



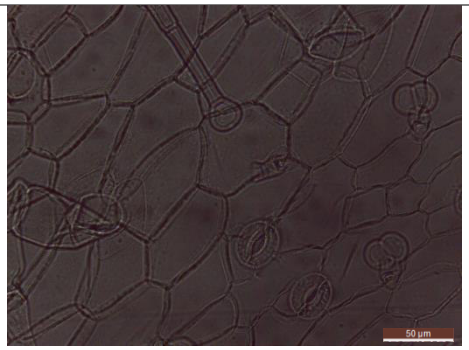
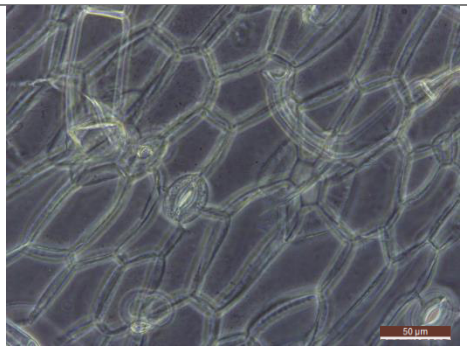
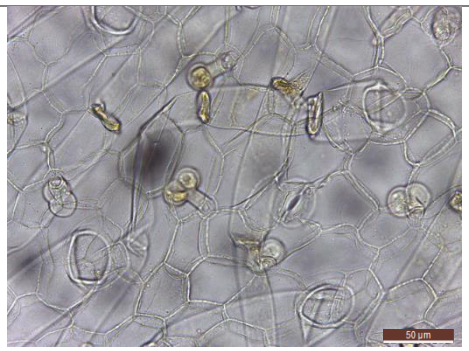
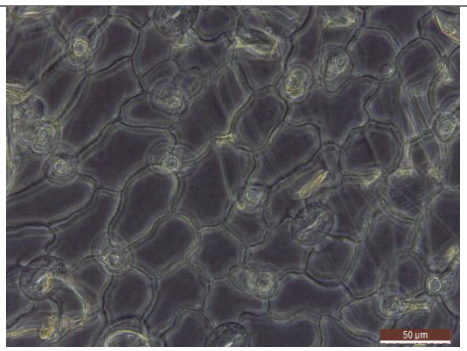
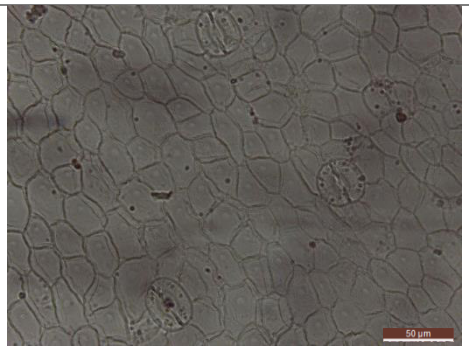
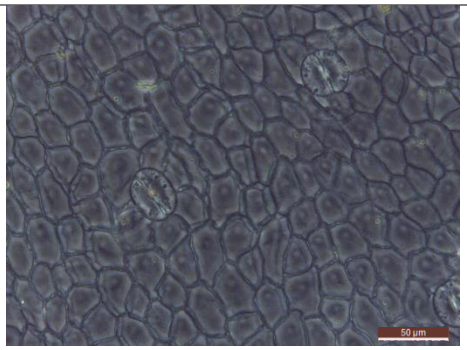
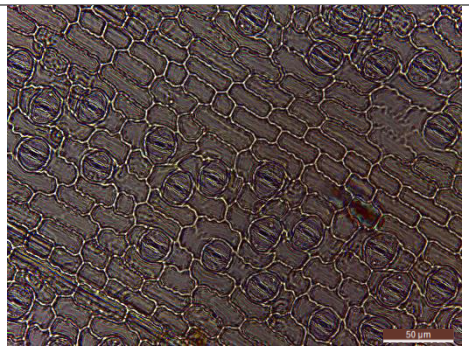
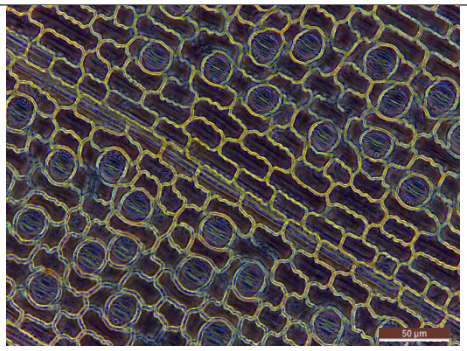
- Flower
(Rectangular cells, medium density, medium size, anomocytic stomata, tector trichomes)

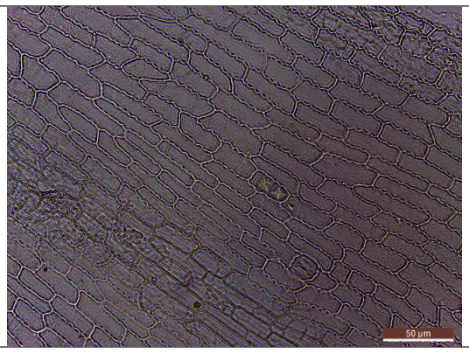
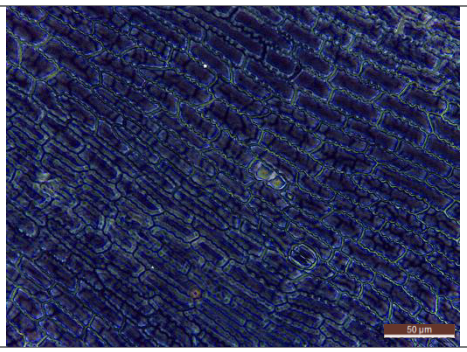
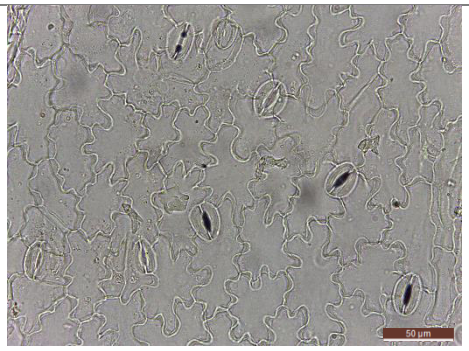
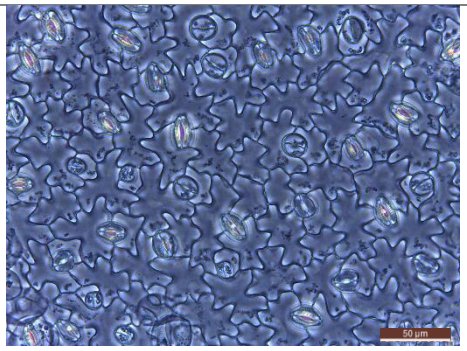
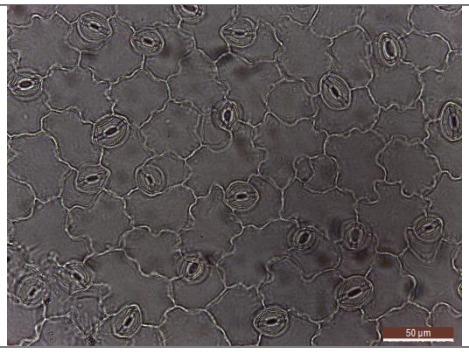
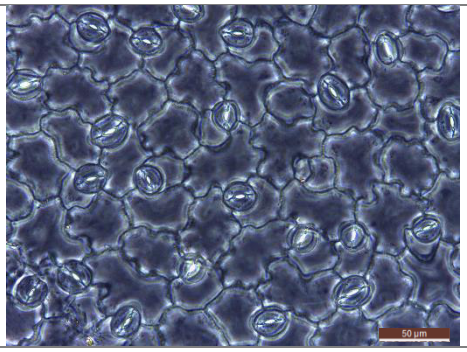
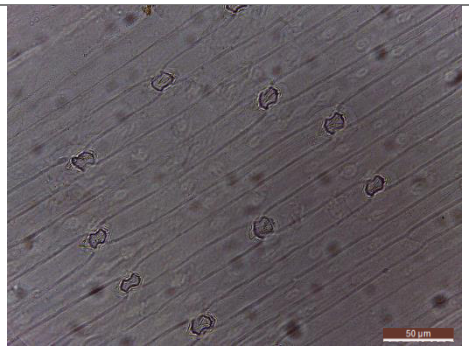
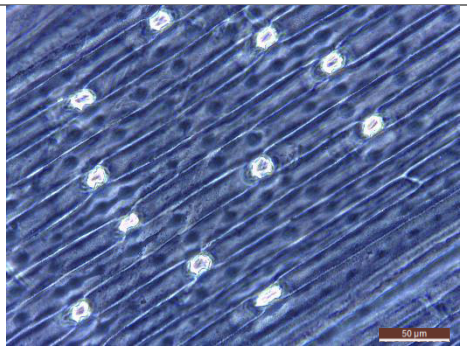



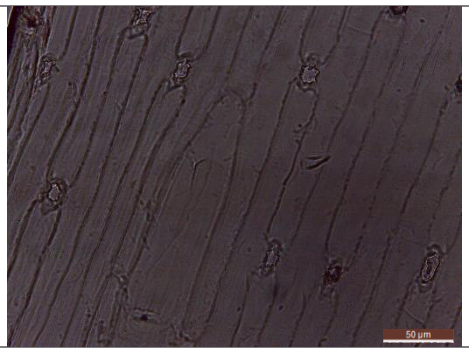
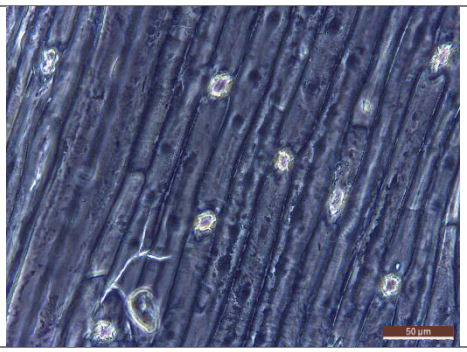
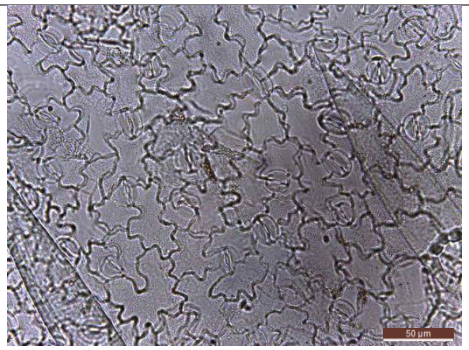
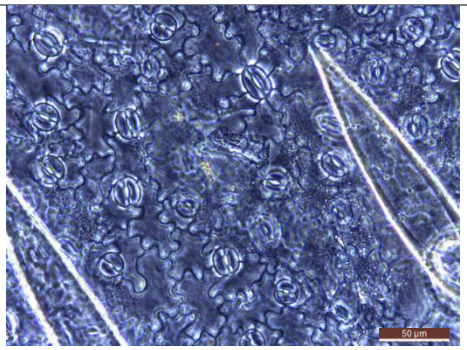
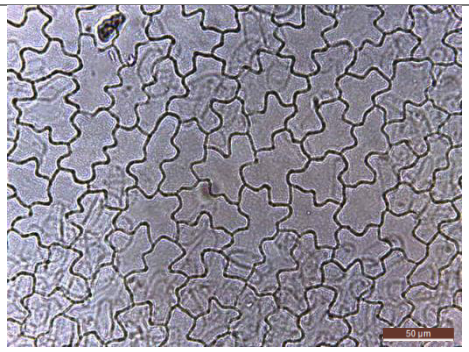
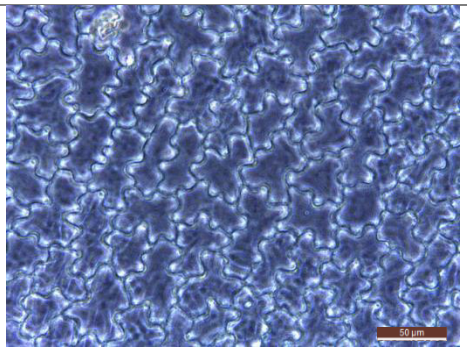
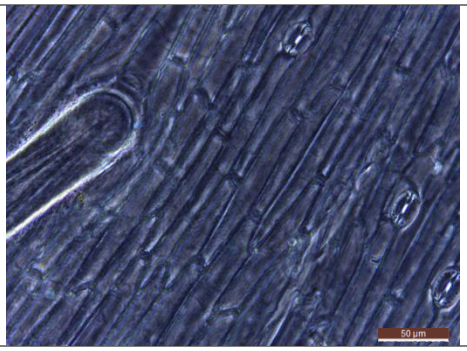
Herbaceous dicotiledons		
Anarrhinum bellidifolium		
- Bottom page (Puzzle cells, low density, big size, anomocytic stomata)		
- Up page (Puzzle cells, low density, big size, anomocytic stomata)		
- Stem (Rectangular cells, low density, big size, anomocytic stomata)		
Athyrium filix-femina		
- Bottom page (Puzzle cells, low density, big size, anomocytic stomata)		

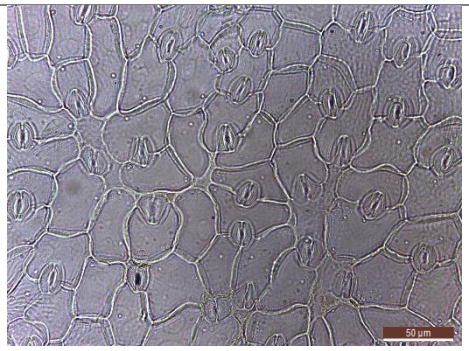
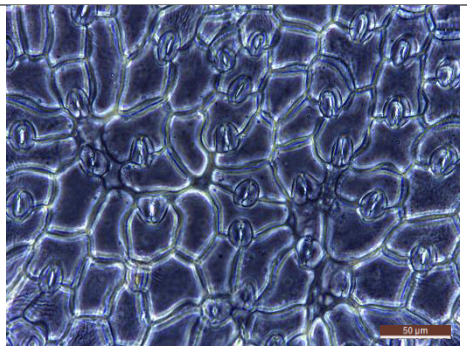
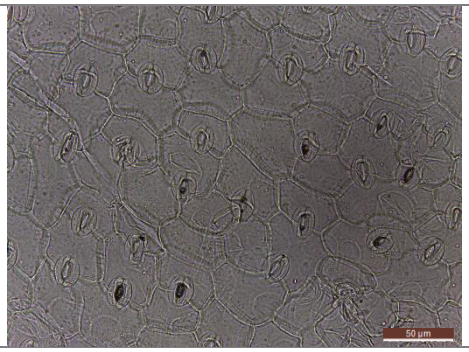
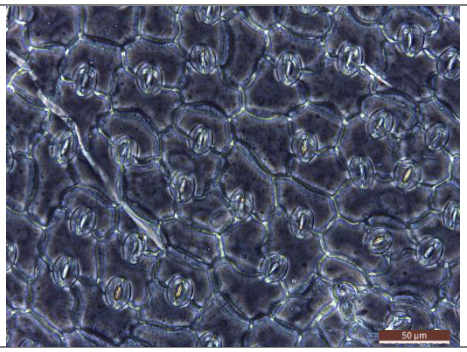
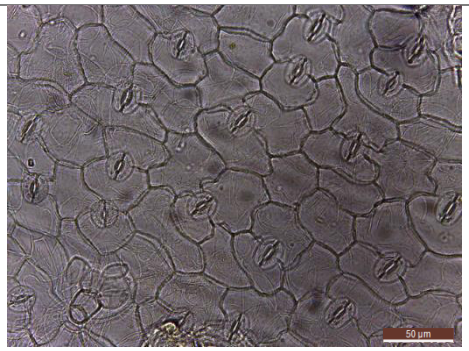
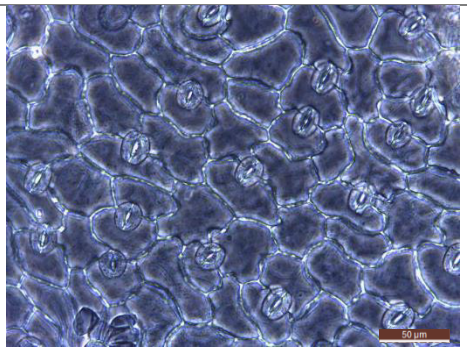
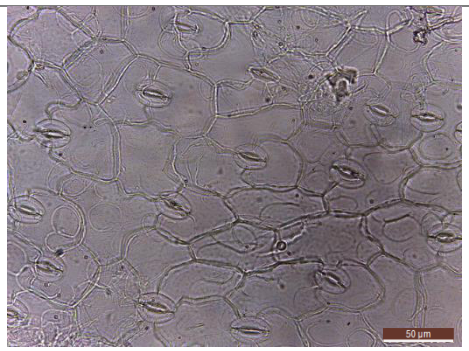
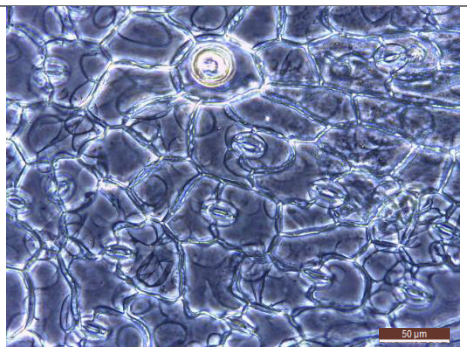
- Up page (Puzzle cells, low density, big size)		
- Stem (Rectangular cells, low density, big size)		
Carduus tenuiflorus		
- Bottom page (Puzzle cells, medium density, medium size, anomocytic stomata, stinging trichomes)		
- Up page (Polygonal cells, low density, big size, anomocytic stomata, stinging trichomes)		

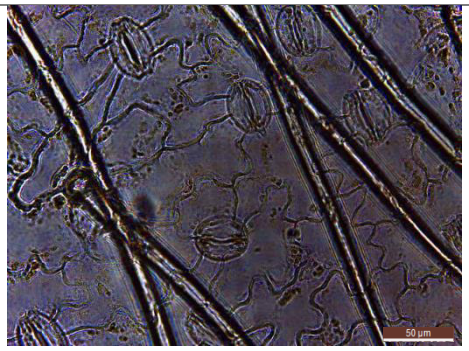
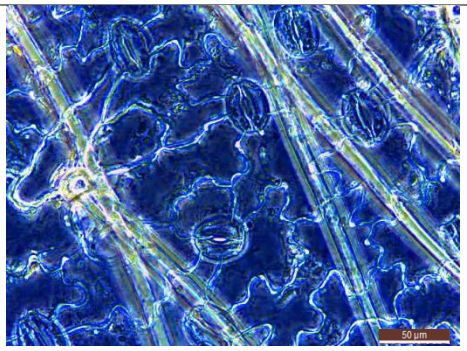
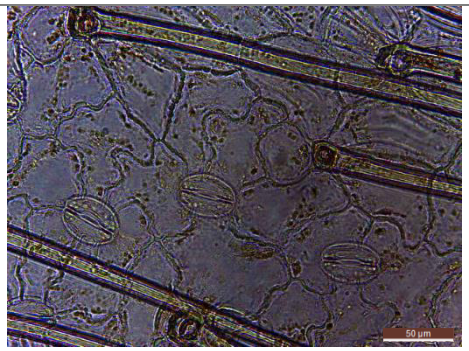
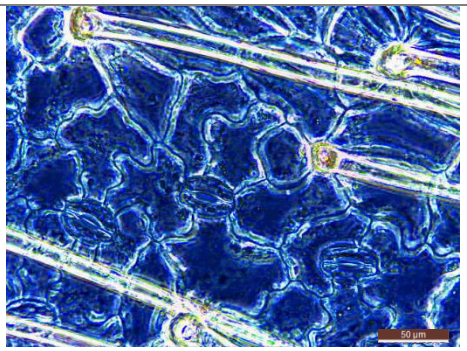
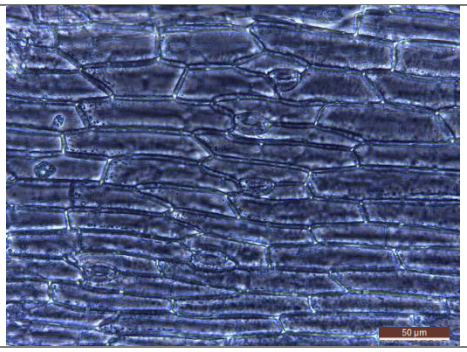
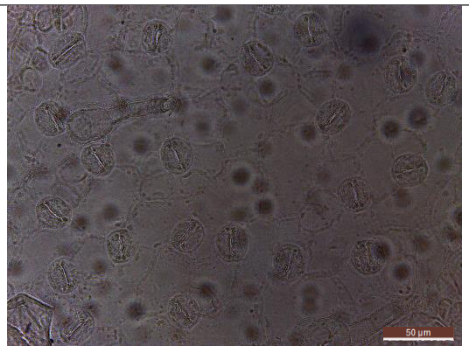
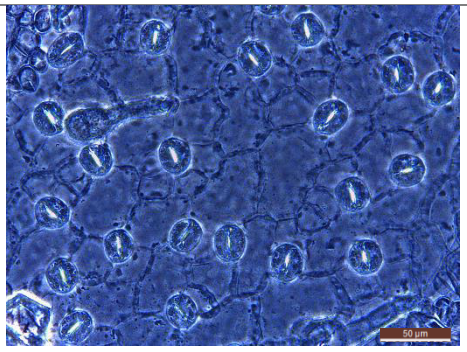
Crepis vesicaria		
- Bottom page (Puzzle cells, medium density, medium size, anomocytic stomata, secretor trichomes)		
- Up page (Puzzle cells, high density, small size, anomocytic stomata)		
- Stem (Rectangular cells, low density, big size, anomocytic stomata)		
Digitalis purpurea		
- Bottom page (Puzzle cells, medium density, medium size, anomocytic stomata, tector trichomes)		

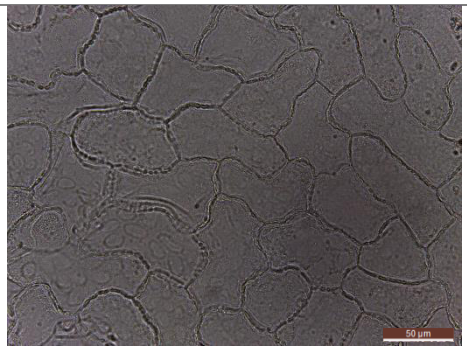
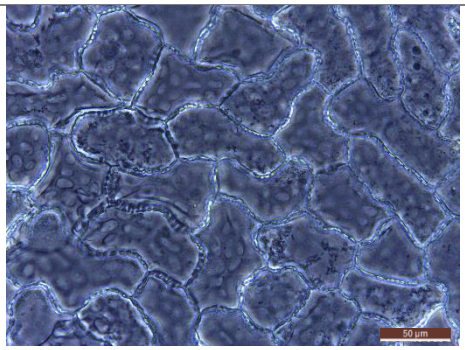
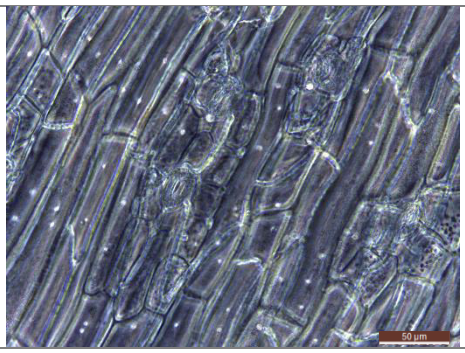
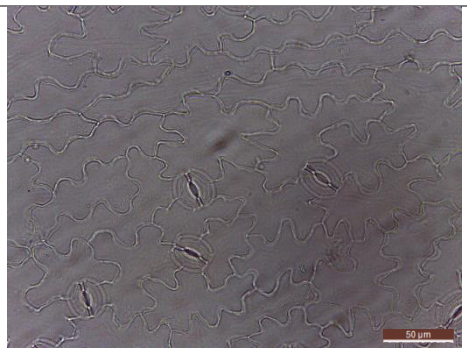
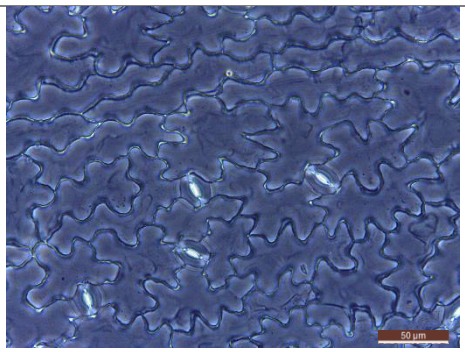
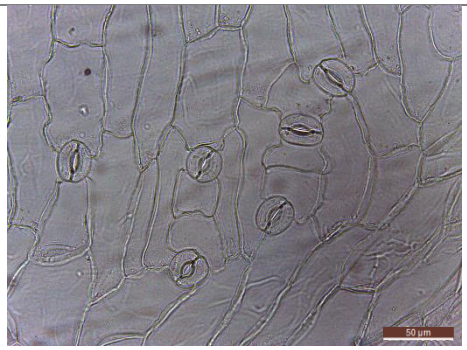
- Up page (Polygonal cells, low density, big size, anomocytic stomata, tector trichomes)		
- Stem (Polygonal cells, low density, big size, anomocytic stomata, tector trichomes)		
- Flower (Polygonal cells, high density, small size, anomocytic stomata)		
Juncus effusus		
- Stem (Rectangular cells, high density, small size, paracytic stomata		

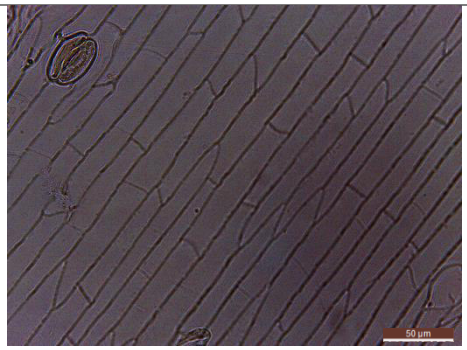
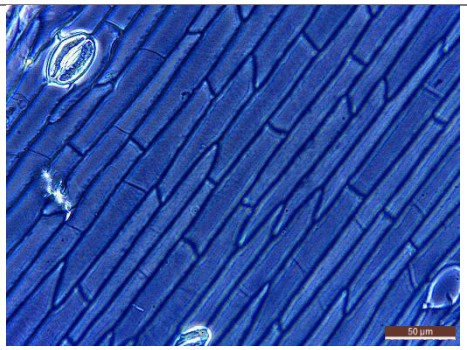
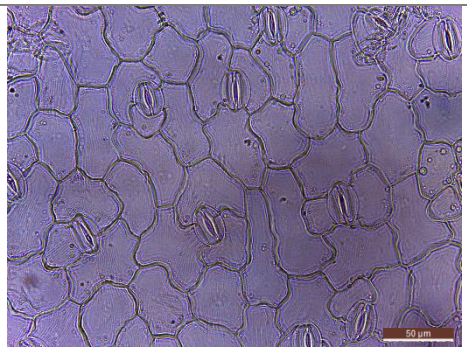
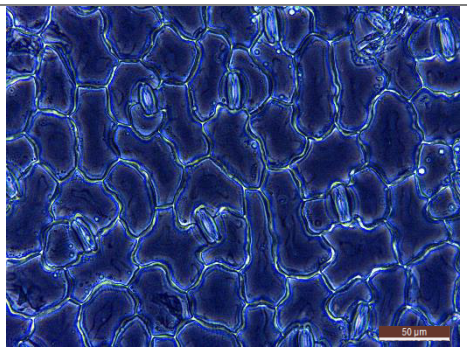
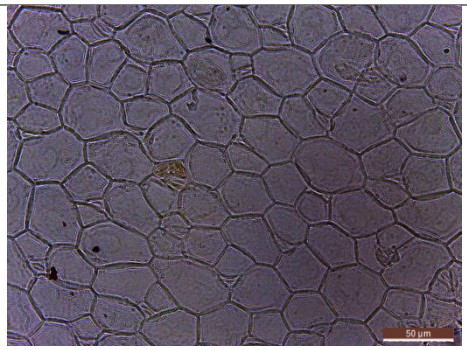
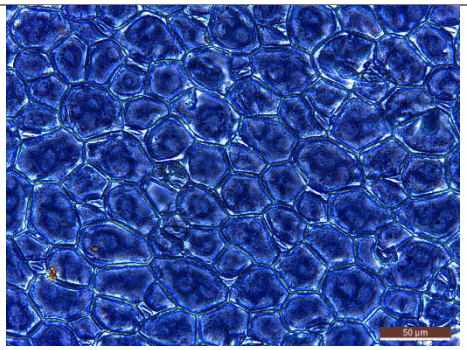
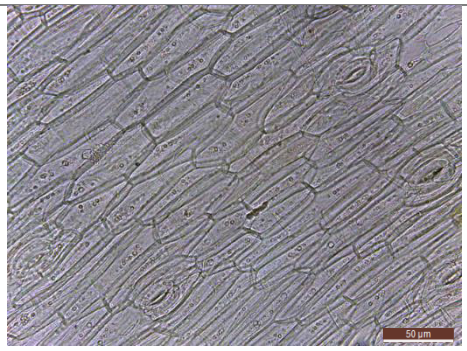
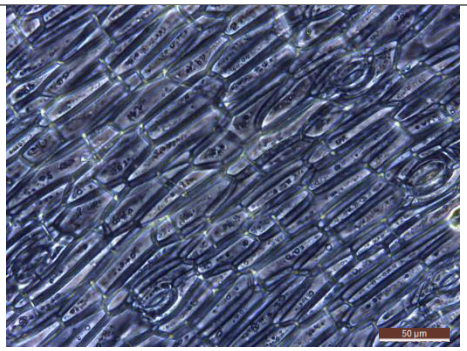
- Flower (Rectangular cells, high density, small size)		
Lepidophorum repandum		
- Bottom page (Puzzle cells, high density, small size, anomocytic stomata)		
- Up page (Polygonal sharply cells, medium density, medium size, anomocytic stomata)		
Narcissus triandrus		
- Bottom page (Rectangular cells, low density, big size, paracytic stomata)		

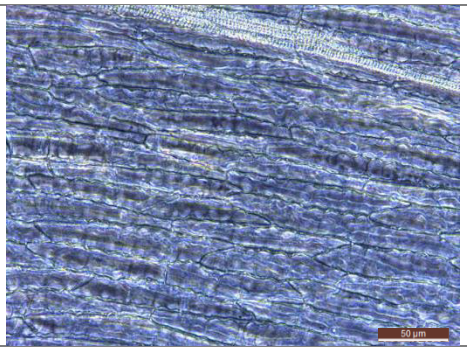
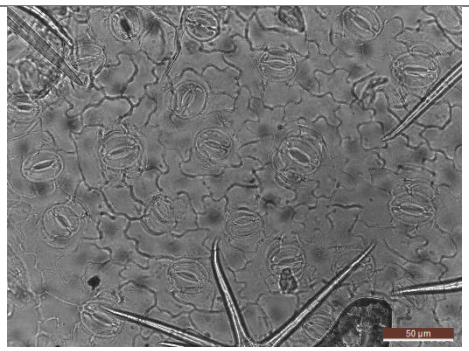
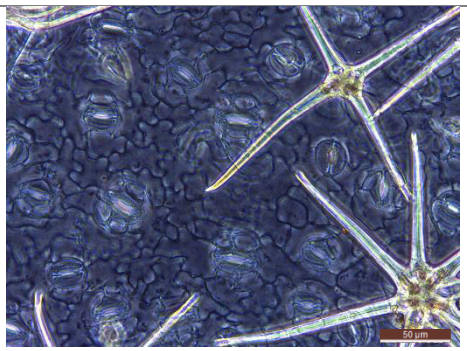
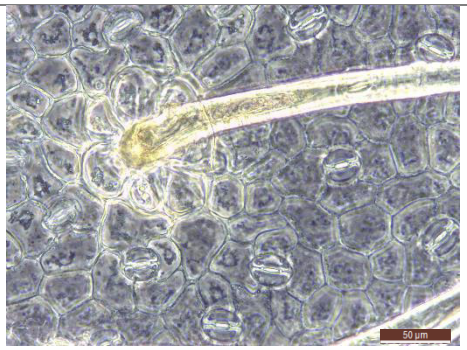
- Up page (Rectangular cells, low density, big size, paracytic stomata)		
Omphalodes nitida		
- Bottom page (Puzzle cells, high density, small size, anomocytic stomata, tector trichomes)		
- Up page (Puzzle cells, high density, small size)		
- Stem (Rectangular cells, medium density, medium size, anomocytic stomata distributed in rows, tector trichomes)		

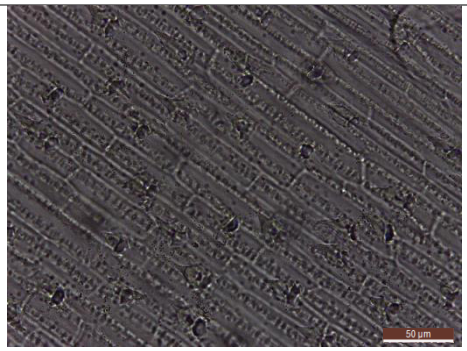
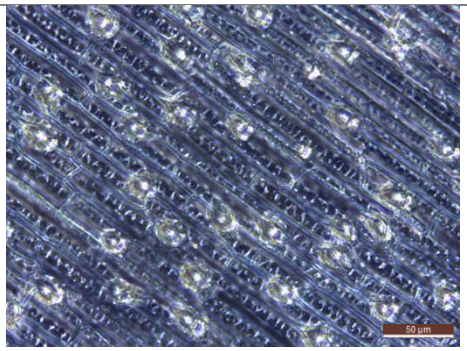
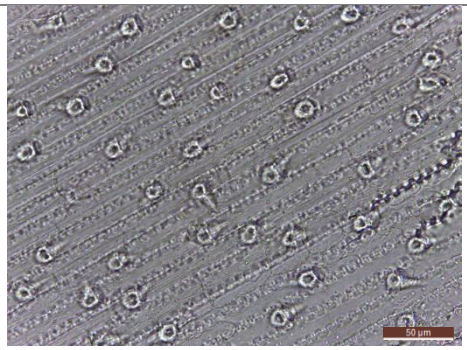
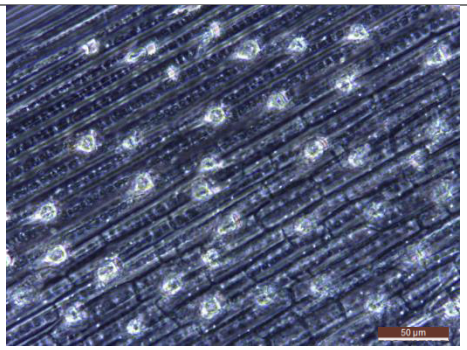
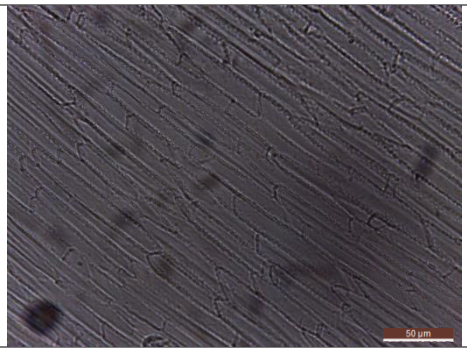
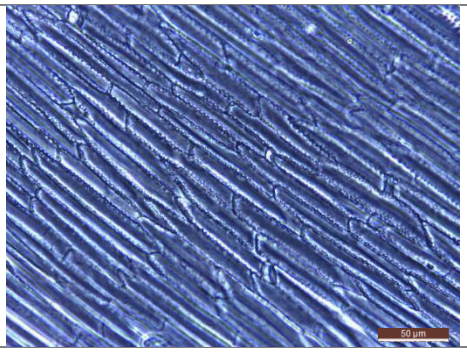
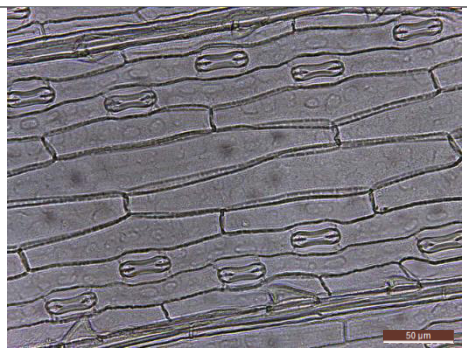
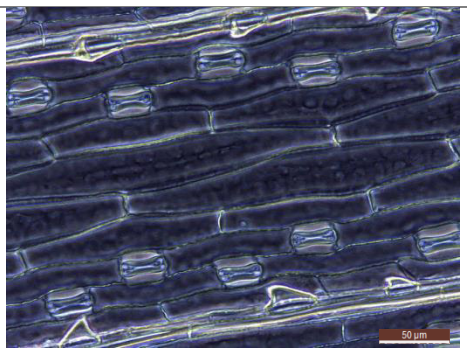
Plantago coronopus		
<u>- Bottom page</u> (Squared cells, medium density, medium size, diacytic stomata)		
<u>- Up page</u> (Squared cells, medium density, medium size, diacytic stomata)		
Plantago lanceolata		
<u>- Bottom page</u> (Polygonal cells, low density, big size, anomocytic stomata, tector trichomes)		
<u>- Up page</u> (Polygonal cells, low density, big size, anomocytic stomata, ttichomes)		

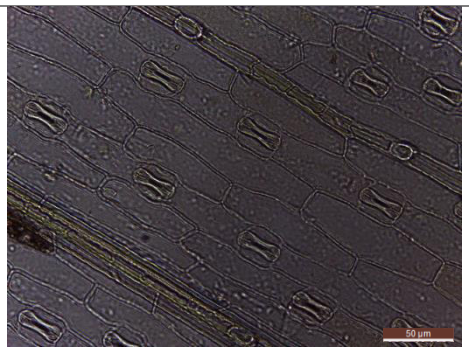
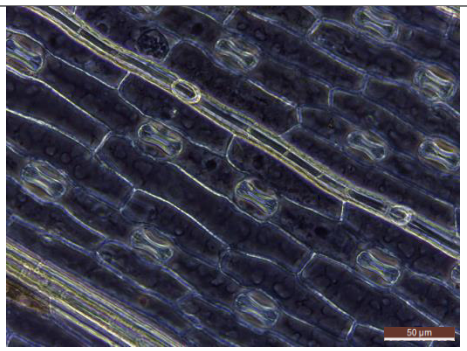
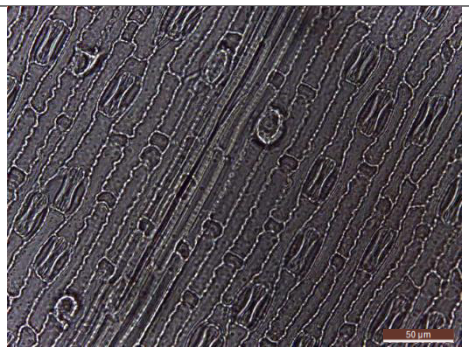
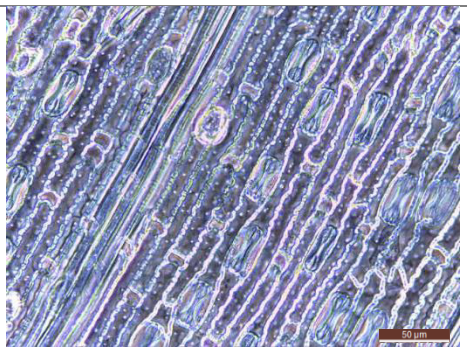
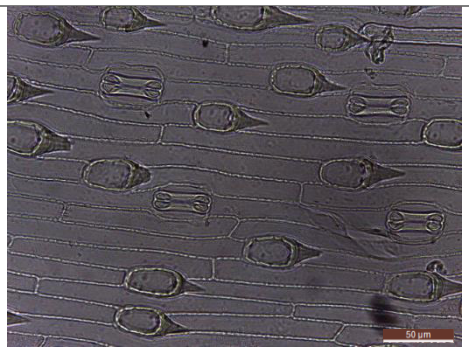
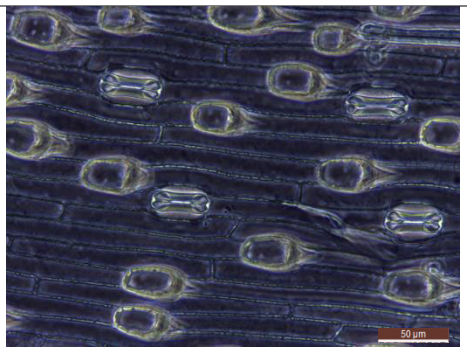
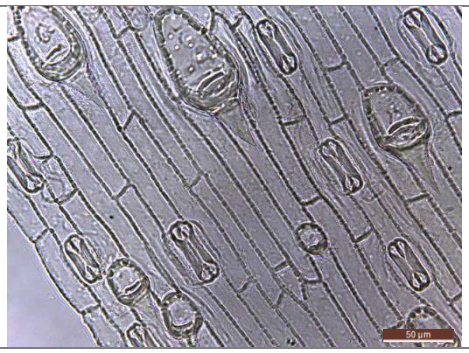
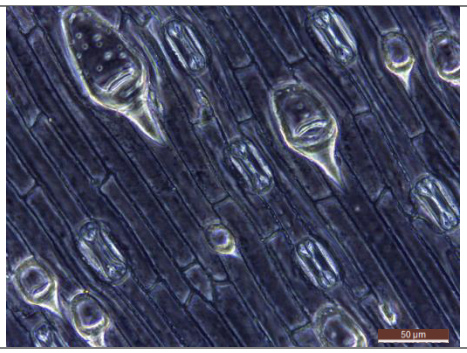
Potentilla erecta		
<u>- Bottom page</u> (Puzzle cells, low density, big size, anomocytic stomata, tector trichomes)		
<u>- Up page</u> (Polygonal sharply cells, low density, big size, anomocytic stomata, tector trichomes)		
<u>- Stem</u> (Rectangular cells, medium density, medium size, anomocytic stomata)		
Sanguisorba minor		
<u>- Bottom page</u> (Polygonal mildly cells, low density, big size, anomocytic stomata)		

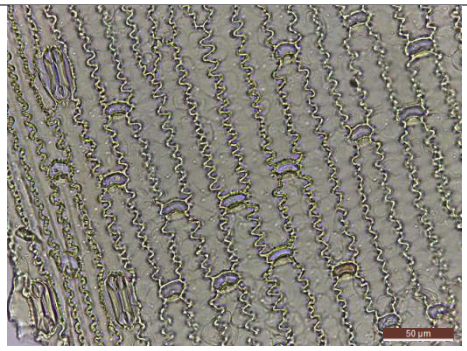
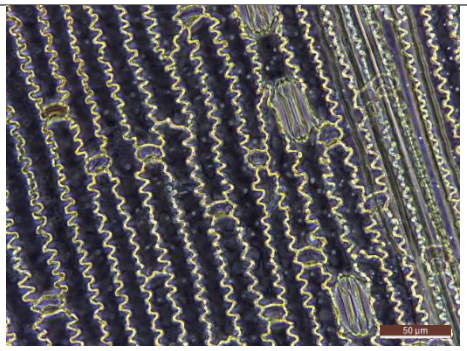
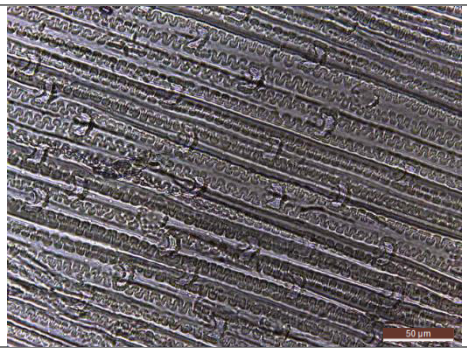
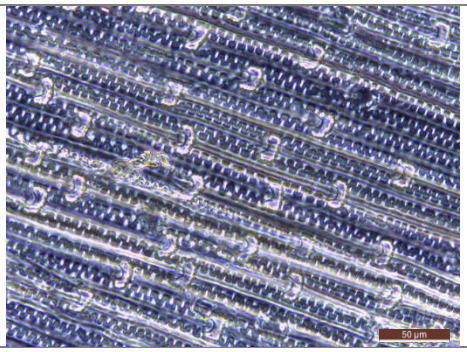
- Up page (Polygonal cells, low density, big size)		
- Stem (Rectangular cells, low density, big size, anomocytic stomata)		
Taraxacum officinale		
- Bottom page (Puzzle cells, medium density, medium size, anomocytic stomata)		
- Up page (Rectangular cells, low density, big size, anomocytic stomata)		

- Stem (Rectangular cells, low density, big size, anomocytic stomata)		
***Trifolium* sp.**		
- Bottom page (Polygonal largely cells, low density, big size, anomocytic stomata)		
- Up page (Polygonal linear cells, high density, small size, rare anomocytic stomata)		
- Stem (Rectangular cells, high density, small size, anomocytic stomata)		

- Flower (Rectangular cells, medium density, medium size)		
Tuberaria lignosa		
- Bottom page (Puzzle cells, high density, big size, anomocytic stomata, starry trichomes)		
- Up page (Polygonal cells, big density, low size, anomocytic stomata, starry trichomes)		

Herbaceous monocotiledons		
Arrhenatherum elatius		
- Leaf (Rectangular cells, low density, big size, stomata in halter, bristles)		
- Stem (Rectangular cells, low density, big size, stomata in halter, bristles)		
- Flower (Rectangular cells, medium density, medium size)		
Dactylis glomerata		
- Bottom page (Rectangular cells, low density, big size, stomata in halter, bristles)		

- Up page (Rectangular cells, low density, big size, stomata in halter, bristles)		
- Stem (Rectangular cells, high density, small size, stomata in halter, bristles)		
- Flower (Rectangular cells, high density, small size, stomata in halter, bristles)		
Hordeum murinum		
- Bottom page (Rectangular cells, low density, big size, stomata in halter, bristles)		
- Up page (Rectangular cells, low density, big size, stomata in halter, bristles)		

- Stem (Rectangular sharply cells, low density, big size, stomata in halter, bristles)		
- Flower (Rectangular sharply cells, low density, big size, bristles)		

Annex 2

Dichotomous key for plant species of the Lousã Mountain

1) A) Polygonal cells ….. 2
   B) Cells with other shapes ….. 43

2) A) Parallel cells ….. 3
   B) Random cells ….. 12
   C) Cells in rosettes ….. **Leaf *Rubus ulmifolius***

3) A) With stomata ….. 4
   B) Without stomata ….. 7

4) A) Covered with trichomes ….. 5
   B) Not covered with trichomes ….. 6

5) A) Anomocytic stomata ….. **Leaf *Acacia dealbata***
   B) Anisocytic, tetracyclic and ciclocytic stomata ….. **Leaf and stem *Genista triacanthos***

6) A) Elongated cells ….. **Leaf *Betula alba***
   B) More or less rounded cells ….. **Leaf *Cupressus lusitanica***

7) A) Covered with trichomes ….. 8
   B) Not covered with trichomes ….. 9

8) A) High density of cells, having approximately 44 µm x 13 µm ….. **Sprout *Betula alba***
   B) Low density of cells, having approximately 47 µm x 28 µm ….. **Leaf *Erica arborea***

9) A) Rounded cells ….. 10
   B) Elongated cells ….. 11
   C) Pentagonal cells ….. **Flower *Erica arborea***

10) A) Cell outline distinct and well defined ….. **Flower *Erica umbellata***

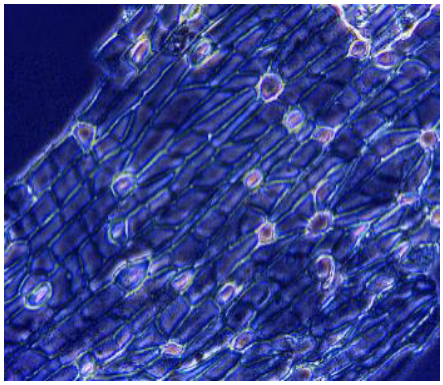
B) Cell outline vague and interlock ….. **Flower *Rubus ulmifolius***

11) A) **Stem *Betula alba***

B) **Stem *Quercus pyrenaica***

C) **Stem *Rubus ulmifolius***

A)



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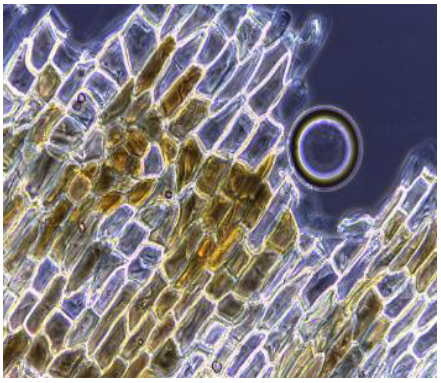

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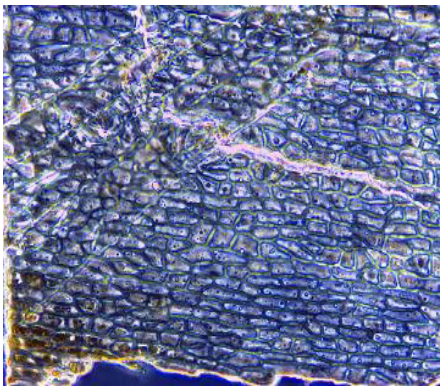


Fig 1: 11. A) Stem *Betula alba*, B) Stem *Quercus pyrenaica*, C) Stem *Rubus ulmifolius*, 400x, phase contrast, Leica application suite V4.6

12) A) With stomata ….. 13

B) Without stomata …. 25

13) A) Covered with trichomes ….. 14

B) Not covered with trichomes ….. 21

14) A) Anomocytic stomata ….. 16

B) Anomocytic and ciclocytic stomata ….. **Bottom page *Cytisus striatus***

C) Anomocytic, ciclocytic and tetracyclic stomata ….. 15

15) A) Stomata more or less in rows, and zones without stomata ….. **Stem *Pterospartum tridentatum***

B) Stomata irregularly distributed ….. **Leaf *Pterospartum tridentatum***

16) A) Tector trichomes ….. 18

B) Starry trichomes ….. 17

C) Stinging trichomes ….. **Up page *Carduus tenuiflorus***

17) A) Cells visibly larger than stomata ….. **Up page *Cistus monspeliensis***

B) Cells having approximately 47 µm x 22 µm ….. **Up page *Tuberaria lignosa***

C) Cells having approximately 34 µm x 23 µm ….. **Leaf *Halimium umbellatum***

D) Cells having approximately 34 µm x 23 µm with visible cellular organization ….. **Stem *Halimium umbellatum***

18) A) Cells with veins ….. **Leaf and stem *Acacia melanoxylon***

B) Cells without veins ….. 19

19) A) Stomata perpendicular to the cell wall and at the junction of 2 to 3 cells ….. **Leaf *Plantago lanceolata***

B) Stomata not as above, randomly distributed ….. 20

20) A) Cell wall undulating sharply and approximately 100 µm x 50 µm with big stomata approximately 42 µm x 22 µm ….. **Up page *Potentilla erecta***

B) Cell wall undulating largely and approximately 60 µm x 51 µm with stomata approximately 28 µm x 27 µm ….. **Leaf *Lithospermum diffusum***

C) Cell wall undulating mildly and approximately 38 µm x 14 µm with approximately stomata 25 µm x 18 µm ….. **Bottom page *Fraxinus* sp.**

D) Cell wall linear ….. 28

21) A) Anomocytic stomata ….. 22

B) Paracytic stomata ….. **Leaf *Eucalyptus globulus***

22) A) Cells with veins ….. 23

B) Cells without veins ….. 24

23) A) Cell wall undulating mildly and approximately 40 µm x 13 µm with stomata approximately 20 µm x 20 µm ….. **Bottom page *Castanea sativa***

B) Cell wall undulating largely and approximately 32 µm x 20 µm with stomata higher than the other cells approximately 44 µm x 32 µm width ….. **Bottom page *Crataegus monogyna***

24) A) Cell wall undulating sharply and approximately 43 µm x 22 µm with high density of stomata approximately 24 µm x 20 µm ….. **Up page *Lepidophorum repandum***

B) Cell wall undulating largely and approximately 69 µm x 30 µm with stomata approximately 30 µm x 22 µm ….. **Bottom page *Trifolium* sp.**

C) Cell wall undulating mildly and approximately 62 µm x 29 µm with stomata approximately 25 µm x 22 µm ….. **Bottom page *Sanguisorba minor***

D) Cell wall linear and approximately 60 µm x 49 µm with stomata approximately 33 µm x 18 µm ….. **Up page *Trifolium* sp.**

25) A) Covered with trichomes ….. 26

B) Not covered with trichomes ….. 36

26) A) Tector trichomes ….. 27

B) Starry trichomes ….. **Leaf *Lavandula stoechas***

27) A) Cells with veins ….. 29

B) Cells without veins ….. 30

28) A) Cells having approximately 77 µm x 24 µm with stomata approximately 37 µm x 28 µm ….. **Up page *Digitalis purpurea***

B) Cells having approximately 60 µm x 26 µm with stomata 35 µm x 27 µm ….. **Stem *Digitalis purpurea***

29) A) Cells having approximately 40 µm x 27 µm ….. **Up page *Quercus pyrenaica***

B) Cells having approximately 26 µm x 18 µm ….. **Leaf *Quercus robur***

30) A) Rounded cells ….. 31

B) Elongated cells ….. 34

C) Cell wall undulating largely …. **Up page *Fraxinus* sp.**

31) A) Intercellular space between the cells ….. **Leaf *Erica australis***

B) Not as above ….. 32

32) A) Big cells, high density of trichomes ….. 33

B) Small cells ….. 34

33) A) Long trichomes ….. **Bottom page *Quercus pyrenaica***

B) Short trichomes ….. **Stem *Lithospermum diffusum***

34) A) Small organization in queue ….. **Stem *Salix atrocinerea***

B) High organization in queue ….. **Leaf *Salix atrocinerea***

35) A) High density of stomata ….. **Sprout *Quercus robur***

B) Very low density of stomata ….. **Stem *Quercus robur***

36) A) Cells with veins …… **Up page *Castanea sativa***

B) Cells without veins ….. 37

37) A) Hexagonal cells ….. **Flower *Erica australis***

B) Elongated cells ….. 38

C) Not as above ….. 39

38) A) Cell wall undulating largely ….. **Flower *Genista triacanthos***

B) Cell wall undulating mildly ….. **Flower *Pterospartum tridentatum***

39) A) Some organization in queue ….. 40

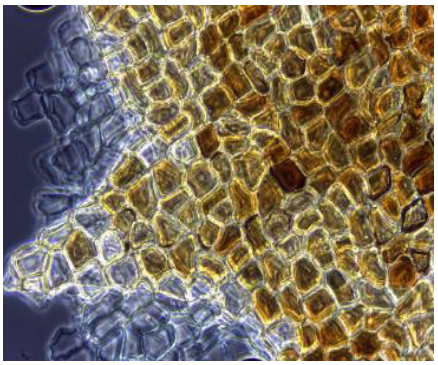
B) Not as above …. 41

40) A) **Stem *Castanea sativa***

B) **Stem *Ilex aquifolium***

C) **Stem *Eucalyptus globulus***

A)



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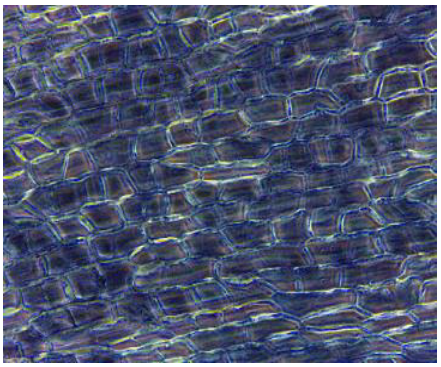

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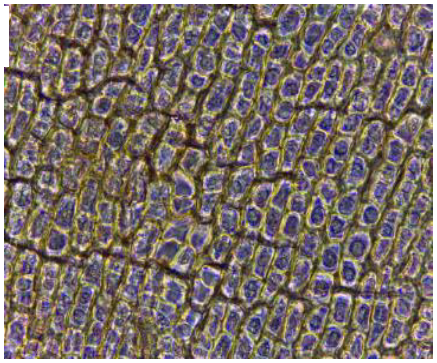


Fig 2: 40.A) *Stem Castanea* sativa, B) *Stem Ilex aquifolium*, C) Stem *Eucalyptus globulus*, 400x, phase contrast, Leica application suite V4.6

41) A) Cell wall undulating largely ….. **Up page *Crataegus monogyna***

B) Cell wall linear ….. 42

42) A) Larger cells, medium density ….. **Sprout *Laurus nobilis***

B) Smaller cells, high density …. **Sprout *Castanea sativa***

43) A) Rectangular cells ….. 45

B) Squared cells ….. 44

C) Puzzle cells ….. 80

44) A) With stomata ….. **Leaf *Plantago coronopus***

B) Without stomata ….. **Stem *Laurus nobilis***

45) A) Parallel cells ….. 49

B) Random cells ….. 46

46) A) With stomata ….. **Up page *Taraxacum officinale***

B) Without stomata ….. 47

47) A) Starry trichomes ….. **Stem *Lavandula stoechas***

B) Tector trichomes ….. 48

48) A) Higher length than width ….. **Sprout *Erica australis***

B) Higher width than length ….. **Stem *Erica australis***

49) A) With stomata ….. 50

B) Without stomata ….. 70

50) A) Covered with trichomes ….. 51

B) Covered with bristles ….. 56

C) Without none of the structures mentioned above ….. 61

51) A) Anomocytic stomata ….. 52

B) Anomocytic and ciclocytic stomata ….. 55

52) A) Higher length than width ….. 53

B) Higher width than length ….. 54

53) A) Stomata randomly distributed ….. **Stem *Cistus monspeliensis***

B) Stomata more or less in rows ….. **Stem *Omphalodes nitida***

54) A) Stomata more or less in rows …. **Stem *Ulex minor***

B) Stomata randomly distributed ….. **Flower *Ulex minor***

55) A) Stomata more or less in rows, and zones without stomata ….. **Up page *Cytisus striatus***

B) Stomata irregularly distributed ….. **Stem *Cytisus striatus***

56) A) Presence of hexagonal cells ….. 57

B) Not as above ….. 58

57) A) Cells having approximately 165 µm x 31 µm and with stomata approximately 37 µm x 26 µm ….. **Bottom page *Dactylis glomerata***

B) Cells having approximately 97 µm x 22 µm and with stomata approximately 36 µm x 26 µm ….. **Up page *Dactylis glomerata***

58) A) Cell wall undulating ….. 59

B) Cell wall linear ….. **Leaf *Hordeum murinum***

59) A) Cell wall undulating sharply ….. 79

B) Cell wall undulating mildly ….. 60

C) Cell wall undulating minutely ….. **Leaf *Arrhenatherum elatius***

60) A) Stomata nearby to the veins ….. **Flower *Dactylis glomerata***

B) Stomata randomly distributed ….. **Stem *Dactylis glomerata***

61) A) Stomata in queue ….. 62

B) Not as above ….. 63

62) A) Two lines of well-defined consecutive stomata ….. **Bottom page *Pseudotsuga menziesii***

b) One line of well-defined consecutive stomata ….. **Leaf *Pinus* sp.**

63) A) Paracytic stomata …. 64

B) Anomocytic stomata ….. 65

64) A) Cell wall undulating mildly, small cells ….. **Stem *Juncus effusus***

B) Cell wall linear, big cells ….. **Leaf *Narcissus triandrus***

65) A) High density of stomata ….. 66

B) Low density of stomata ….. 67

66) A) Stomata more or less in rows ….. **Leaf *Ulex minor***

B) Stomata randomly distributed ….. **Stem *Anarrhinum bellidifolium***

67) A) Some hexagonal cells ….. **Stem *Trifolium* sp.**

B) Not as above ….. 68

68) A) Rectangular structure defined by right angles ….. **Stem *Potentilla erecta***

B) Not as above ….. 69

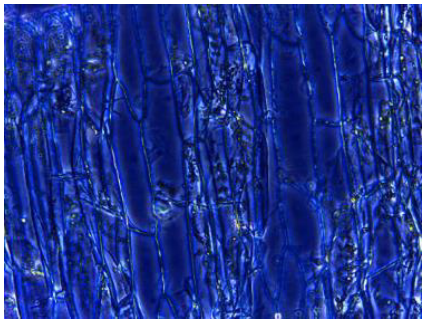
69) A) **Stem *Crepis vesicaria***

B) **Stem *Sanguisorba minor***

C) **Stem *Taraxacum officinale***

D) **Stem *Crataegus monogyna***

A)



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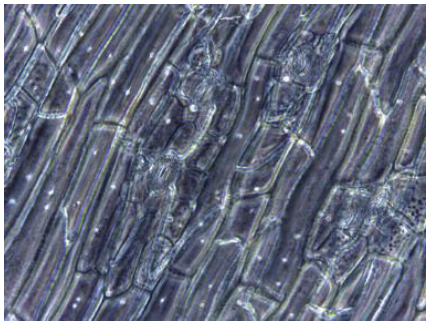

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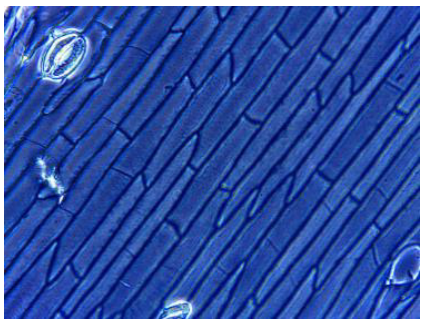

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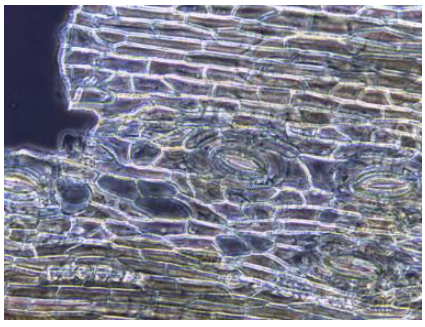


Fig 3: 69.A) Stem C*repis vesicaria*, B) Stem *Sanguissorba minor*, C) Stem *Taraxacum officinale*, D) Stem *Catraegus monogyna*, 400, phase contrast, Leica application suite V4.6

70) A) Covered with trichomes ….. 71

B) Covered with bristles ….. 78

C) Without none of the structures mentioned above …..74

71) A) Cell wall undulating ….. **Leaf *Erica umbellata***

A) Cell wall linear ….. 72

72) A) Short trichomes ….. **Stem *Pseudotsuga menziesii***

B) Thorn-like trichomes ….. 73

73) A) Rectangular structure defined by right angles ….. **Stem *Erica umbellata***

B) Not as above …. **Stem *Erica arborea***

74) A) Cell wall undulating ….. 75

B) Cell wall linear ….. 76

75) A) Cell wall sharply ….. **Flower *Cistus monspeliensis***

B) Cell wall mildly ….. **Flower *Trifolium* sp.**

76) A) Higher length than width ….. 77

B) Higher width than length ….. **Leaf and stem *Chamaecyparis lawsoniana***

77) A) **Up page *Pseudotsuga menziesii***

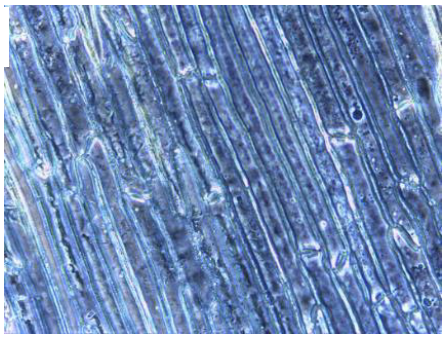
B) **Sprout *Pseudotsuga menziesii***

C) **Stem *Pteridium aquilinum***

D) **Stem *Athyrium felix-femina***

E) **Stem *Fraxinus* sp.**

A)



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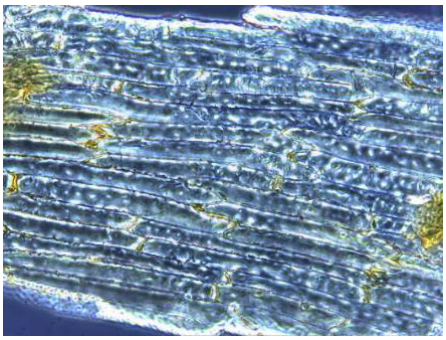
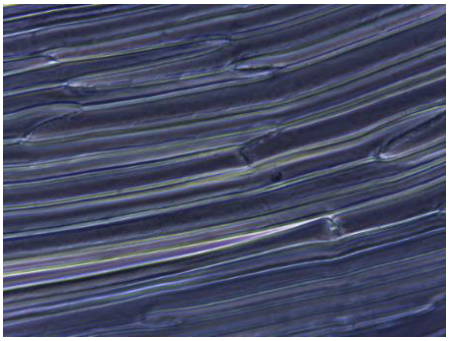


C)

D)



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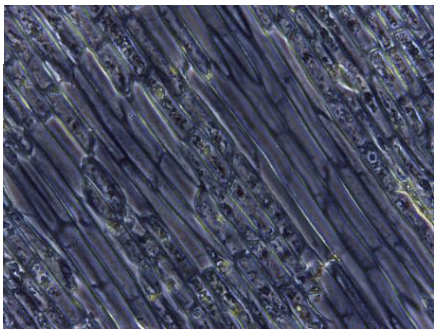

Fig 4: 77.A) Up page *Pseudotsuga menziesii*, B) Sprout *Pseudotsuga menziesii*, C) Stem *Pteridium aquilinum*, D) Stem *Athyrium felix-femina*, E) Stem *Fraxinus* sp., 400x, phase contrast, Leica application suite V4.6

78) A) Cell wall undulating sharply ….. **Flower *Hordeum murinum***

B) Cell wall undulating minutely ….. **Flower *Arrhenatherum elatius***

79) A) Smaller cells, having approximately 132 µm x 18 µm ….. **Stem *Hordeum murinum***

B) Larger cell, having approximately 171 µm x 19 µm ….. **Stem *Arrhenatherum elatius***

80) A) With stomata ….. 81

B) Without stomata ….. 92

81) A) Covered with trichomes ….. 82

B) Not covered with trichomes ….. 87

82) A) Stinging trichomes ….. **Bottom page *Carduus tenuiflorus***

B) Secretory trichomes ….. **Bottom page *Crepis vesicaria***

C) Starry trichomes ….. 83

D) Tector trichomes ….. 84

83) A) Cell wall both undulating sharply and largely ….. **Bottom page *Cistus monspeliensis***

B) Cell wall only undulating sharply ….. **Bottom page *Tuberaria lignosa***

84) A) With veins ….. **Bottom page *Pteridium aquilinum***

B) Without veins ….. 85

85) A) Long trichomes ….. 86

B) Short trichomes ….. **Bottom page *Omphalodes nitida***

86) A) Larger cells ….. **Bottom page *Potentilla erecta***

B) Smaller cells ….. **Bottom page *Digitalis purpurea***

87) A) Ciclocytic stomata ….. **Bottom page *Ilex aquifolium***

B) Paracytic stomata ….. **Bottom page *Laurus nobilis***

C) Anomocytic stomata ….. 88

88) A) With veins ….. **Bottom page *Athyrium felix-femina***

B) Without veins ….. 89

89) A) Some organization in queue ….. **Up page *Anarrhinum bellidifolium***

B) Not as above ….. 90

90) A) Larger cells ….. 91

B) Smaller cells ….. **Bottom page *Lepidophorum repandum***

91) A) Cells having approximately 66 µm x 25 µm with stomata mostly in the interface of three cells having approximately 22 µm x 18 µm ….. **Bottom page *Anarrhinum bellidifolium***

B) Cells having approximately 87 µm x 33 µm width stomata mostly in the interface of four cells ….. **Bottom page *Taraxacum officinale***

92) A) With veins ….. 93

B) Without veins ….. 94

93) A) Higher cells, without organization ….. **Up page *Athyrium felix-femina***

B) Smaller cells with organization in queue ….. **Up page *Pteridium aquilinum***

94) A) Cell wall undulating mildly ….. **Stem *Pinus nigra***

B) Cell wall undulating sharply ….. **Up page *Laurus nobilis***

C) Cell wall undulating largely ….. 95

95) A) Higher dimensions, cells having approximately 75 µm x 25 µm ….. **Up page *Omphalodes nitida***

B) Smaller dimensions, cells having approximately 41 µm x 21 µm ….. **Up page *Ilex aquifolium***

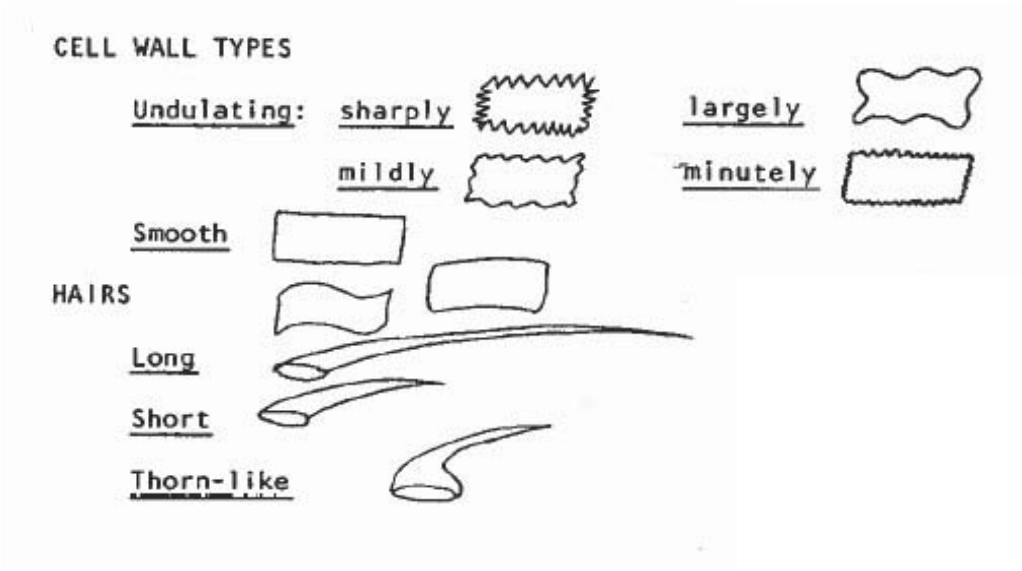

Fig 5: Cell wall and trichomes types used to compare the cells (adapted from Longhurst et al, 1979)