SAINFOIN (*ONOBRYCHIS VICIIFOLIA*), A FORAGE LEGUME WITH GREAT POTENTIAL FOR SUSTAINABLE AGRICULTURE, AN INSIGHT ON ITS MORPHOLOGICAL, AGRONOMICAL, CYTOLOGICAL AND GENETIC CHARACTERISATION

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Life Sciences

2011

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ABSTRACT

The University of Manchester
Christine Hayot Carbonero
A thesis submitted for the Degree of Doctor of Philosophy

Sainfoin (*Onobrychis viciifolia*), a forage legume with great potential for sustainable agriculture, an insight on its morphological, agronomical, cytological and genetic characterisation

March 2011

Sainfoin (*Onobrychis viciifolia*), is a traditional forage legume whose agricultural use has been in constant decrease in Western Europe since the 1960's. However, growing evidence suggests that it may be of great interest in the context of sustainable agriculture, thanks to numerous beneficial properties (nutritional, environmental and anthelmintic). In the frame of a large project network, an extensive *O. viciifolia* (and other *Onobrychis* species) germplasm has been gathered and several accessions were grown in small plots on an experimental field at NIAB, Cambridge. Measurements of morphological and agronomical traits were performed on these plots. Cytological and molecular genetics studies were also carried on the germplasm.

Accessions were found to be highly variable in their agronomical traits, with differences in productivity. It was observed that *O. viciifolia* was relatively resistant to diseases, but that persistence was the main difficulty to overcome. *O. viciifolia* accessions were also found to be variable in their morphological traits.

Statistical analyses on both morphological and agronomical traits showed strong links with accessions' geographic origins. The most important trend observed is a general distinction between Western European accessions and accessions from the rest of the world.

It was found that most *O. viciifolia* were tetraploids, suggesting that agricultural domestication led to polyploidy. Other *Onobrychis* species were found to be either diploid or tetraploid with varying basic chromosome numbers, which tends to confirm the assumption that an aneuploidy event occurred in *Onobrychis* genetic history.

AFLP and SSR fingerprinting were attempted to investigate *O. viciifolia* genetic diversity. The potential of these techniques was shown, but the latest improvements needed to obtain solid data were not achieved during this study. Still, it was shown that molecular marker assisted breeding programmes can be elaborated for *O. viciifolia*.

Phylogenetic analyses were performed through sequencing of different DNA regions. Substantial genetic diversity was observed among *O. viciifolia* accessions, with again a general distinction between Western European accessions and accessions from the rest of the world. A clarification of the *Onobrychis* genus is suggested, as it appeared that many species must be synonyms, and that many taxonomic sections are weakly supported.

Overall, it appeared that *O. viciifolia* potential has probably not been fully exploited, and that there is an important potential for improvement in the gemplasm studied here. Due to its superiority in animal husbandry and agroecologic impact, *O. viciifolia* improvement can be suggested as a valuable alternative to extensively used forage legumes.

DECLARATION

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ACKNOWLEDGMENT

These investigations were supported by the European Commission (Project: Marie Curie Research Training Network-CT-2006-035805).

I would like to thank my supervisors Dr Lydia Smith and Professor Terry Brown for their support in the course of my researches. Special thanks also go to my NIAB project colleagues: Dr Ilya Gadjev (from Bulgaria), Joan Doidy (from France), Laura Falco Sales (from Spain), Guillaume Barbier (from France) and Nadeesha Bandara (from Sri Lanka) for their help in the project duties and support with my PhD work.

I would like to thank some HealthyHay team leaders for sharing ideas and organising useful workhops: Dr. Irene Mueller Harvey (University of Reading, UK), Professor Karl Stich and Dr. Heidi Halbwirth (TUW, Austria), Fernando Munoz and Dr. Ignacio Delgado (CITA, Spain), Professor Dieter Treutter (TUM, Germany), Jocelyne Aufrere (INRA Theix, France), Herve Hoste (INRA Toulouse, France), Dr. Wilbert Pellikaan (WU, the Netherlands), Ian Wilkinson and Robin Hill (Cotswold Seeds, UK). I also would like to thank my other HealthyHay colleagues for sharing this project with its numerous meetings and workshops, participating to NIAB duties and/or exchanging ideas: Samir Demdoum (from France worked in CITA, Spain), Martin Lorenz (from Austria worked in SLU, Sweden), Mila Domenis (from Italy worked in WU, the Netherlands), Dr. Ana Castro (from Portugal worked in TUW, Austria), Angelika Galezowska (from Poland worked in University of Reading, UK and in TUW, Austria), Marina Dobreva (from Bulgaria worked in University of Reading, UK), Elisabetta Stringano (from Italy worked in University of Reading, UK), Dr. An Gea (from Belgium worked in University of Reading, UK), Jana Thill (from Germany worked in TUW, Austria), Justyna Kusek (from Poland worked in TUW, Austria), Asma Ahmad (from Pakistan worked in TUW, Austria), Ionela Regos (from Romania worked in TUM, Germany), Koraljka Paskas (from Croatia worked in TUM, Germany), Katerina Theodoridou (from Greece worked in INRA Theix, France), Fotini Manolaraki (from Greece worked in INRA Toulouse, France), Celia Arroyo (from Spain worked in INRA Toulouse, France), Anastasios Saratsis (from Greece, worked for NAGREF, Greece), Dr. Adam Novobilsky (from Czech Republic worked for Royal Veterinary and Agricultural University, Danemark), Bayissa Hatew (from Ethiopia worked for WU, the Netherlands).

I would also like to thank some NIAB colleagues that participating in my work progress and training: Dr. David Lee, Dr. Huw Jones, Dr. Ian Mackay, Steven Bentley, Dr. James Cockram, Richard Lockwood, Dr. David Kenyon, Linda Mail, Richard Horsnel, Dr. Fiona Leigh, Dr. Nick Gosman, Dr. Alison Bentley, David Smith and Claire Pumfrey.

I also would like to thank other people that permitted me to progress: Dr. Jaroslav Dolezel (Institute of Experimental Botany, Czech Republic), Dr. Peter Civan (University of Manchester, UK) and Dr. Peter Isaac (IDna Genetics Ltd, UK).

Finally, the biggest thank go to my husband Dr. Franck Carbonero, who gave me the help and energy to complete my PhD work and enrich my everyday life.

LIST OF ABBREVIATIONS

AFLP: Amplified fragment length polymorphism
AM: Arbuscular mycorrhizal
bp: Base pair
BSA: Bundessortenamt
CAP: Common agricultural policy
Cd: Intergenic spacer between *trnT* and the 5' exon of *trnL*
CITA: Centro de Investigacion y Technologia Agroalimentaria
CTAB: Cetyl-trimethylammonium bromide
DAPI: 4', 6-diamidino-2-phenylindole
ddNTP: Dideoxyribonucleotide triphosphate
DUS: Distinctness uniformity and stability
DM: Dry matter
dNTP: Deoxynucleoside triphosphate
G1 phase: Period in the cell cycle during which neither cell division nor preparation for cell division occurs
G2 phase: Period occurring between the DNA replication and the various steps of mitosis
GRIN: Germplasm Resources Information Network
ICARDA: International Center for Agricultural Research in the Dry Areas
IGER: Institute of Grassland and Environmental Research
ITS: Internal transcribed spacer
LAI: Leaf area index
NAS: National Academy of Sciences
NGA: National Geospatial Intelligence Agency
NIAB: National Institute of Agricultural Botany
O.: Onobrychis
OTU: Operational Taxonomic Unit
P: Probability
PCR: Polymerase chain reaction
PI: Propidium iodide
PhD: Doctor of Philosophy
PsbA: Intergenic spacer between *trnH* and *PsbA* gene
R: Correlation coefficient
RAC: Royal Agricultural College
RBG: Royal Botanic Gardens
RCAH: Research Centre for Agrobotany
REML: Restricted maximum likelihood
RICP: Gene Bank Research Institute of Crop Production
Sp.: Species
SSR: Simple sequence repeat
Tm: Melting temperature
UPOV: International Union for the Protection of New Varieties of Plants
UK: United Kingdom
UV: Ultraviolet

CHAPTER 1. INTRODUCTION

In many parts of Europe, the cultivation of forage legumes has decreased since the early 1980's. This was mainly due to the impact of support payments from the Common Agricultural Policy towards intensive production; farmers were encouraged to use more inorganic nitrogen fertilisers. This trend is now changing, however, and pressure to reduce energy consumption, environmental pollution and improve agricultural sustainability is getting stronger. This is now encouraging farm businesses to have a more responsible attitude to the environmental impact of their activities, by using low-input systems including the use of forage legumes (Rochon *et al.*, 2004). Recent literature has shown that systems based on forage legumes have the ability to positively impact on the environment. Forage legumes have been shown to increase efficiency of nitrogen use and decrease nitrogen transit from the soil. Moreover, global warming is projected to increase the yield of forage legumes, relative to grasses (Frame *et al.*, 1998). From the perspective of livestock nutrition, recent studies have demonstrated that forage legumes with moderate levels of secondary compounds, such as condensed tannins and flavonoids, are beneficial. In particular, they increased the efficiency of nitrogen utilisation in the digestive tract, reduced bloat hazard and decreased parasitism. These beneficial attributes might convince farmers to cultivate more forage legumes with these properties such as sainfoin.

1.1. Sainfoin: a perennial forage legume from the *Onobrychis* genus

Sainfoin (*Onobrychis viciifolia*) is a perennial forage legume, an important group in agriculture. This group also includes widely cultivated crops such as lucerne (*Medicago sativa*), white clover (*Trifolium repens*) and red clover (*Trifolium pratense*) but also less common species such as birdsfoot trefoil (*Lotus corniculatus*). Legumes are able to convert inorganic gaseous atmospheric nitrogen into bioavailable nitrogen compounds (ammonium) thanks to a symbiotic association with bacteria (the symbiont species of bacteria can be from

several genera). Ammonium is then directly usable by the plants to produce proteins (Frame *et al.*, 1998). *O. viciifolia* may be used as both forage and fodder, which means that it is eaten fresh by grazing animals and that it can be harvested for feeding animals.

1.1.1. Botanical description

O. viciifolia is an erect or sub-erect plant. It grows from 40 to 100 cm in height (Thomson, 1951a; Frame *et al.*, 1998). Many hollow stems, arising from basal buds, form a branched crown. Each stem has pinnate leaves formed with 10 to 28 leaflets grouped in pairs on long petioles and with a terminal leaflet. The stipules are broad and finely pointed. The inflorescences develop on axillary tillers with about 80 pinkish red melliferous flowers. Each flower can produce a kidney-shaped seed contained in a brown pod. The fruit is either spiny or spineless (Figure 1). The degree of spininess is characteristic for different lines and is genetically determined (Thomson, 1951b). The size of the true seeds is variable from 2.5 to 4.5mm long, 2 to 3.5mm broad and 1.5 to 2mm thick. The weight per thousand unmilled seed and milled seed is approximately 24 grammes and 15 grammes, respectively. The fruit colour is determined by the ripeness at harvesting time. A deep taproot with a few main branches and numerous fine lateral roots form the root system. *O. viciifolia* is an outbreeding species mostly pollinated by insects. It is self incompatible.

O. viciifolia has been divided into two agricultural types termed ‘Giant Sainfoin’ and ‘Common Sainfoin’ and there are a number of key differences between the two types. The common type (*Onobrychis sativa* var. *communis* (Ahlefed)) is from central Europe. Its growth habit remains prostrate in the year of sowing and regrowth after the first spring cut is slow and vegetative. The aftermath is normally grazed. It is also named single-cut sainfoin due to this limitation in terms of regrowth.

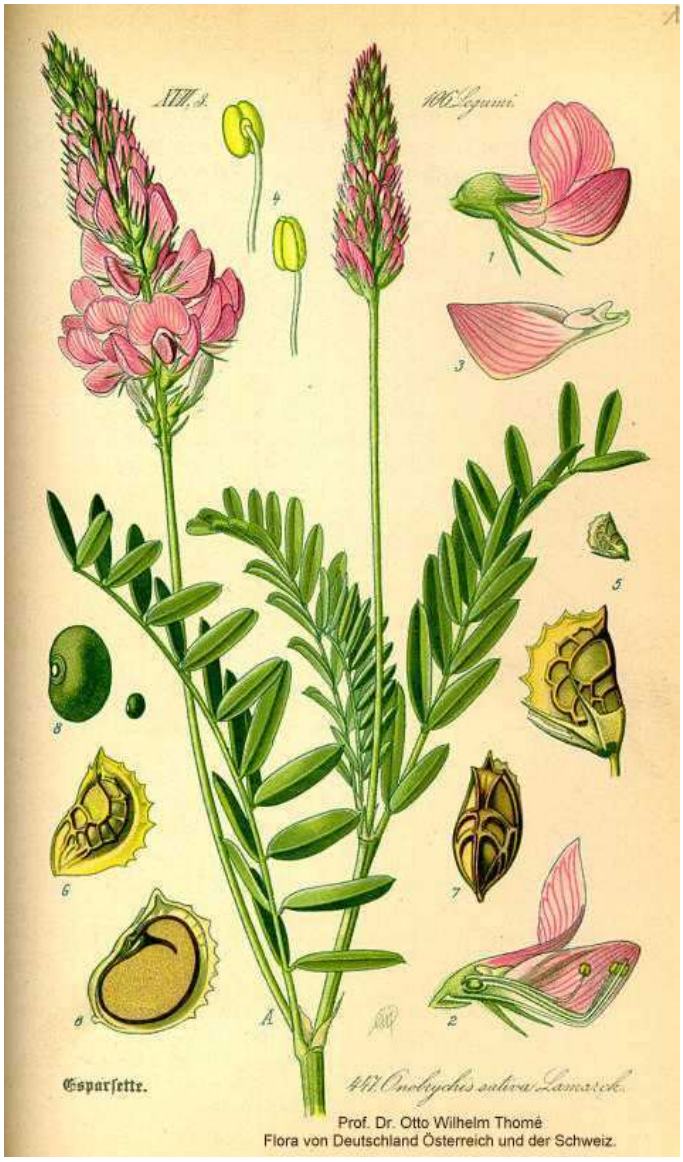

Figure 1: *Onobrychis viciifolia* (by permission from *www.biolib.de*)

The Giant type or double-cut sainfoin (*Onobrychis sativa* var. *bifera* Hort.) is from the Middle East. It grows more quickly into an erect habit during the first year of growth. It has the ability to reflower after being cut (Badoux, 1965). Most importantly from the farmer's point of view, it can be cut more than once per year, but unlike common types, which will persist for 10 years or more, the giant types will not normally survive beyond three years. The giant type has proportionally less stem per plant, longer stems and more internodes per stem. It also has more leaflets per leaf than the common type (Thomson, 1951a). There are no other relevant differences of particular note between the 'Giant' and the 'Common' types relating to seed weight, colour and spininess of the unmilled

fruit (Thomson, 1951a). Negri and Cenci (1988) characterised twenty populations of *O. viciifolia* from central Italy. They found that the several populations have their own morphological characters mainly related to the altitude. At greater altitude, populations were mainly characterised by: generally reduced dimensions of the vegetative parts, leaflets having a rounder shape, prostrate growth habit, shorter peduncle of inflorescence and a greater length of inflorescence.

1.1.2. Cytological aspects of *Onobrychis viciifolia*

O. viciifolia is reported to be either a diploid or a tetraploid species with respectively 2n=2x=14 and 2n=4x=28 chromosomes (Frame *et al.*, 1998). However, El-enain (2002) discusses the occurrence of series of 2n=22, 27, 28 and 29 chromosomes (2n=3x+1, 4x-1, 4x, 4x+1), which demonstrates the role of aneuploid alteration from the chromosome number based on multiples of x=7 in the evolution of this species.

Only scarce information can be found on diploid *O. viciifolia*. Most literature only refers to tetraploid *O. viciifolia* with 2n=4x=28 (Kidambi *et al.*, 1990b). Negri *et al.* (1987), analysed the ploidy of 20 different populations which were all tetraploid. Tamas (2006), studied cytology aspects of *O. viciifolia* and concluded that it is a tetraploid species with average chromosome lengths being 3.39μm in general, 3.79μm for the longest and 1.6μm for the shortest.

1.1.3. *Onobrychis* genus taxonomy

Sainfoin belongs to the genus *Onobrychis*, which belongs to the tribe *Hedysareae* of the subfamily *Papilionoideae* of the fabaceae family, previously called leguminosae. *Onobrychis* is one of the most difficult genera to deal with. Many confusions and contradictions are found in the taxonomy of *Onobrychis*. This is mostly due to different approaches in species delimitation resulting in a varying number of recognised species (Emre *et al.*, 2007). Yildiz *et al.* (1999), suggested that the genus *Onobrychis* comprises circa 170 species, based on fruit

morphology. They are classified into two subgenera *Sisyrosema* and *Onobrychis*, and 8 sections. Guner *et al.* (2000), estimated that 54 species can be identified in the genus *Onobrychis* and that they are divided into 5 sections. Širjaev (1925), suggested the classification of *Onobrychis* species shown below in Table 1.

Table 1: Classification of *Onobrychis* adapted from Širjaev (1925)

Genus	**Subgenus**	**Sections**
Onobrychis	*Euonobrychis=Onobrychis*	Dendrobrychis
		Lophobrychis
		Hemicyclobrychis
		Eubrychis=Onobrychis
	Sisyrosemae	Anthyllium
		Afghanicae
		Heliobrychis
		Hymenobrychis

The most widespread species of this genus is *O. viciifolia* (Celiktas *et al.*, 2006). Several Latin names are used in the literature for sainfoin: *Hedysarum onobrychis* L., *Onobrychis sativa* Lam., *Onobrychis viciaefolia* Scop and *Onobrychis viciifolia* Scop.

Sánchez-Yélamo (2006) characterised some species of the genus *Onobrychis* using isozyme methods. *Onobrychis* species section Eubrychis clustered in a main group. The taxa belonging to subsections *Hispanicae, Brachysemiae* and *Macropterae* clearly appear differentiated from the species of subsection *Vulgatae*.

Emre *et al.* (2007) suggested a classification based on total seed protein profiles. Studied species of sections Lophobrychis, Onobrychis, Hymenobrychis clustered together.

Ahangarian *et al.* (2007), clarified the phylogeny of *Onobrychis* genus based on nrDNA ITS sequences. It has been suggested recently that subgenus *Sisyrosemae* was derived from subgenus *Onobrychis* (Ahangarian, 2007).

1.2. History of *Onobrychis viciifolia* cultivation

O. viciifolia was traditionally cultivated in many parts of the world but in the last century its culture has been declining. Its English name 'sainfoin' is derived from the French 'sain foin', which means 'healthy hay'. *O. viciifolia* is also known as cock's head, holy grass, esparcette and French grass.

1.2.1. Distribution

O. viciifolia has been cropped for hundreds of years in many parts of the world, including Asia, Europe and North America (Frame *et al.*, 1998).

O. viciifolia is native to South Central Asia and was introduced into central Europe in the fifteenth century (Burton and Curley, 1968). It was first cultivated in Southern France in 1582, following which it spread over Europe (Piper, 1924). It was introduced to North America in 1786, but was only occasionally cultivated until the 1960's, when improved varieties allowed wider cultivation in adapted areas, primarily Montana and parts of Western Canada. Today, *O. viciifolia* is still being cropped mainly in Eastern Europe, Italy, Spain, Iran and Turkey. It seems especially popular in Turkey, where about 94,000 ha were reportedly grown in 2001 (Eken *et al.*, 2004)..

The date of its first introduction to the United Kingdom has not been documented accurately, but its use was reported in the 17th century by Hartlib (1652). *O. viciifolia* was cropped in the 17th, 18th, 19th and early 20th century in many areas of Britain. Its very high quality hay was used to feed the heavy working horses and the aftermath grazing was preferred for fattening lambs (Koivisto and Lane, 2001).

1.2.2. The decline of *Onobrychis viciifolia* in Western Europe

Over the last 40 years, *O. viciifolia* has experienced a constant decline in

Europe (Borreani *et al.*, 2003). It has been recorded that more than 150 tonnes of seeds were sold every year in the late 1950s in the United Kingdom, enough for 2,500 hectares (Hill, 1998). In the late 1970s only approximately 150 hectares were cropped, and this number continued to decrease again after this time.

Doyle *et al.* (1984) assessed the future economic potential of *O. viciifolia* in United Kingdom agriculture. It was estimated that *O. viciifolia* could potentially be grown on 950,000 hectares in England and Wales, where the soil is sufficiently alkaline. To be more widely grown, it was suggested that the *O. viciifolia* yield should be increased by 35%, which would yield circa 11.5 t DM ha^{-1}. Under experimental conditions, yields of about 14-16 t DM ha^{-1} have been achieved (Sheehy *et al.*, 1984) in the United Kingdom, which indicates the potential for achieving 12 t DM ha^{-1} yield in practical farming.

Today, *O. viciifolia* has become rare in the United Kingdom, being grown by only a few farmers. Hutchinson (1965) suggested that the cause of this decline might have been due, in part, to its poor response to the changing requirements and circumstance of British agriculture. Rochon *et al.* (2004) also pointed out that the decline of forage legumes in Europe has been due to the farmers support payments towards intensive production using cheap inorganic fertilisers since the early 1970s. Hill (1998) further explained that this may also have been due to the expansion and dominance of autumn cereal cropping from the 1960s. Borreani *et al.* (2003) explained its decline in Italy as a result of agricultural structural changes, and the gradual disappearance of livestock farms in hilly areas. Newman (1997) stated that the virtual disappearance of *O. viciifolia* was mainly due to the replacement of hardworking draught horses by tractors, for which it was a major feed.

Agronomic problems however, may be the main cause of decline since *O. viciifolia* is reported to be of low yield, low persistence and poor regrowth after the first cut, compared to *Medicago sativa* (Sims *et al.*, 1968; Borreani *et al.*, 2003).

1.3. Technical knowledge on *Onobrychis viciifolia*

O. viciifolia has been the subject of a range of studies relating to agronomy, environmental preferences, crop protection and plant-microbial interactions.

1.3.1. Climate and soil requirements

O. viciifolia is adapted to a wide range of climatic conditions e.g. in Europe, North America, Asia, Australia and New Zealand, to neutral and alkaline soils of pH 6 or above, and also to dryland and irrigated areas, similar to *Medicago sativa*. In the United Kingdom, it has always been linked with calcareous chalky or limestone soil where it has been reported as growing well (Frame *et al.*, 1998). *O. viciifolia* is intolerant of water-logging and so the soil needs to be well drained (Sheldrick *et al.*, 1987). Studies conducted at the Grassland Research Institute, Hurley, showed that *O. viciifolia* formed a thin and patchy sward on clay with pH below 6, and that there were failures on alluvial sand with pH below 5 in the Thames Valley (Bland, 1971). Light or medium soil with pH 6 or above without waterlogging seems therefore, to be preferred for *O. viciifolia*.

Meyer and Badaruddin (2001) have compared the frost tolerance of several legume species by using young seedlings. The forage legumes were the most tolerant to freezing temperatures. *O. viciifolia* seedlings were more resistant than *Medicago sativa* and most of the *Trifolium* species. Only *Trifolium hybridum* seedlings were more resistant.

1.3.2. Sowing

O. viciifolia seeds are sold in two forms. The first, termed “unmilled seed” or “seed in husk”, consists of the whole one-seeded fruits. The other form, termed “milled seed”, is comprised of only the true seed with the dry husk removed (Thomson, 1951b). There are conflicting reports in the literature

concerning the relative germination of the two forms. Milled seed is reported to have better germination than unmilled seed (Wiesner *et al.*, 1968), but Chen (1992) reported that there was no significant difference in emergence in the field between the two forms. It has been also reported by Cash and Ditterline (1996) that seedlings from large seed were stronger. They had more nodules and higher rates of nitrogen fixation at most harvest dates up to 84 days. Therefore, it would appear that using seeds of a minimum size is important for breeding purposes.

In the United Kingdom, *O. viciifolia* sowing normally takes place between April and July when the soil is warm enough for rapid seed germination and there is sufficient moisture for absorption by the seed. Early spring sowing allows the crop a longer vegetative period to develop strong roots and shoots, and possibly even to give a harvest in the establishment year. A sowing date at any time between April and July was found to give similar yield the following season, whereas an August and September sowing reduces the forage yield in the following season (Liu and Lane, 2005).

Jensen and Sharp (1968) reported that the optimal temperature for germination was between 10 and 20° C; and advised that 5°C was the minimum temperature to sow. The seeds should be drilled or broadcast to a depth of 1 to 2 centimetres according to traditional experience (Hill, 1998). Canadian experience indicates that the optimum depth was less than 2 centimetres (Goplen *et al.*, 1991) but Chinese experience suggested 4 to 5 centimetres (Chen, 1992). These different recommendations for optimum sowing depth probably reflect differences in soil texture and moisture availability but also probably considering the variety being grown.

Goplen *et al.* (1991) recommended seeding rate and row spacing from 7 kg ha^{-1} and 60 cm for seed production to 40 kg ha^{-1} and 15 cm for irrigated hay production. Density trials conducted in a greenhouse at Grassland Research Institute, Hurley, indicated that 100 plants m^{-2} produced the maximum *O. viciifolia* yield in the establishment year and suggested a optimum seed rate of about 62.5 kg ha^{-1} assuming 80% germination (Sheehy *et al.*, 1984).

1.3.3. Mixed sward of *Onobrychis viciifolia* with other species

In general, *O. viciifolia* mixtures with certain grass species yield more than each component of the mixture alone (Dubbs, 1968). A number of authors advise the use a companion crop for *O. viciifolia* to avoid invasion and thus competition from weeds (Koivisto and Lane, 2001). Goplen *et al.* (1991) recommended a reduction in the seeding rate of each species to about two-thirds of that recommended for pure stands. Traditionally, *O. viciifolia* was sown with a non-aggressive companion grass such as meadow fescue (*Festuca pratensis*) and timothy (*Phleum pratense*) (Frame *et al.*, 1998). In Montana, *O. viciifolia* was mixed with Kentucky bluegrass (*Poa pratensis*), red fescue (*Festuca rubra*), black medic (*Medicago lupulina*), *Trifolium repens* and *Lotus corniculatus*. The *O. viciifolia-L. corniculatus* mixture was the most compatible and productive (Cooper, 1972). Liu *et al.* (2006) recommended that the best yield was obtained from a mixture 2/3 of *O. viciifolia* and 1/3 of *F. pratensis*.

1.3.4. Weed control

O. viciifolia is usually considered to be a non-aggressive crop with slow regrowth after cutting, requiring it to be established with minimum competition from weeds. Weeds can have a crucial effect on *O. viciifolia* production in the establishment year. In the case of *O. viciifolia* grown without herbicides, weeds comprised 98% of the total yield for the first cut in the establishment year (Moyer, 1985). Traditionally the addition of *Festuca pratensis* or *Phleum pratense* was a means to avoid weed ingress. Alternatively, undersowing *O. viciifolia* in spring barley may also suppress weeds during establishment.

In the United Kingdom, weeds in *O. viciifolia* crops sown in the spring are mainly broad leaf species such as cleavers (*Galium aparine*), fat hen (*Chenopodium album*), groundsel (*Senecio vulgaris*) and red dead-nettle (*Lamium purpurum*) and in the autumn chickweed (*Stellaria media*) is often severe.

A range of herbicides have been used to control the several types of weeds. To successfully control most spring germinating broad leaf weeds, MCPA [a.i. 4-(4-Chloro-2-methyl-phenoxy) acetic acid] and MCPB [a.i. 4-(4-Chloro-2-methyl-phenoxy) butyric acid] have been applied in practice at the first trifoliate stage of *O. viciifolia*. Carbetamine [$(R)^{-1}$-(ethylcarbamoyl) ethylcarbanilate] has been applied in winter successfully to maintain *O. viciifolia* swards free from grass weeds and chickweed (Sheldrick and Thomson, 1982; Frame *et al.*, 1998).

1.3.5. Inoculation, nitrogen fixation and fertilisation

O. viciifolia forms symbioses of two types: with rhizobia bacteria and with mycorrhizal fungi. The plant benefits from increased sequestration of certain essential mineral nutrients such as nitrogen and phosphate.

1.3.5.1. Biology of symbioses

O. viciifolia develops two types of symbioses; firstly the development of specialist organelles termed nodules with a range of *Rhizobium sp.*, which enable fixation of gaseous nitrogen into inorganic molecules. Second symbioses, known as mycorrhiza, are formed with with a range of fungus species and are associated with increased phosphate sequestration among other attributes.

1.3.5.1.1. Rhizobia-Legume plant symbiosis: the nodule

A symbiotic interaction can occur between Gram-negative bacteria of the family *Rhizobiaceae* and legume plants' roots. A specialist organ, the nodule is then formed. Various forms of nodule exist ranging from spherical, to branched and coralloid. In this nodule, differentiated bacteria (bacteroids) use a nitrogenase enzyme complex in order to reduce atmospheric nitrogen to ammonia. The plant then uses this ammonia in order to synthesise amino acid and protein. In return, the plant supplies the rhizobia with the products of

photosynthesis. The infection by rhizobia occurs generally through root hairs. The interaction between rhizobia and host shows a high degree of specificity. Therefore, the successful infection of the roots by rhizobia is dependant upon a reciprocal molecular dialogue between the host plant and the rhizobia. However, a single host species may be nodulated by several different genera and species of bacteria. The bacteria in the nodules vary from essentially parasitic to highly effective in delivering ammonia (Sprent, 2003). The root-nodule bacteria entering in symbiosis with the genus *Onobrychis* belong to the genera *Mesorhizobium*, *Rhizobium* and *Bradyrhizobium* (Baimiev *et al.*, 2007).

1.3.5.1.2. Arbuscular mycorrhizal (AM) symbiosis

The AM symbiosis formed between plant roots and fungi is one of the most widespread symbiotic associations found in plants. 80% of vascular flowering plants are able to form this type of symbiosis with fungi, which are members of the zygomycetes. The AM association is relatively non-specific, highly compatible and long lasting. The plant supplies the fungus with carbon. The fungus assists the plant with the acquisition of phosphate and other nutrients from the soil and also influences the plants resistance to invading pathogens. The interaction begins when fungal hyphae, arising from spores or adjacent colonised roots, contact the root surface. Then they differentiate to form appressoria via which they penetrate the root. The fungus, once inside the roots, may grow both inter and intracellularly throughout the cortex but does not invade the vasculature or the meristematic region. In addition, the fungus also maintains external mycelia which ramify out into the soil in order to access phosphate. The phosphate is then transported into the internal structures within the plant root and eventually made available to the plant accross the fungal/plant interface (Harrison, 1998).

1.3.5.2. Inoculation

O. viciifolia can be cross-inoculated by *Rhizobium* species from sweet vetch (*Hedysarum* sp.), crownvetch (*Coranilla* sp.), and purple and white prairie

clover (*Dalea purpurea* and *Dalea candida*) (Burton and Curley, 1968). *O. viciifolia* can also be inoculated with rhizobia isolated from three arctic legume species: *Astragalus alpinus, Oxytropis maydelliana* and *Oxytropis arctobia*. This inoculation with arctic rhizobia species improves nitrogen fixation during cold phases of the growing season (Prevost *et al.*, 1987).

Mycorrhizal inoculation has not been studied on *O. viciifolia*. However, AM are known to help plants to access phosphate but also to be one of the most efficient ecological factors in improving growth and N content in a legume (Barea and Azcon-Aguilar, 1983). A study on sulla (*Hedysarum coronarium*), showed that AM improved nitrogen fixation thanks to the phosphate supply and also enhanced nitrogen uptake from the soil (Barea *et al.*, 1987).

1.3.5.3. Nitrogen fixation

Overall, nitrogen fixation rates of *O. viciifolia* have been measured to be within the range of other forage legumes (Liu *et al.*, 2006). In comparison to other legumes, the nitrogen fixation rate of *O. viciifolia* has been measured in terms of both the amount of nitrogen fixed and expressed in terms of resultant increase in yield. For *O. viciifolia* the rate in most situations was between 130 and 160 kg ha^{-1}, compared to 140-210 for *M. sativa*, this resulted in an increase in yield of 17 and 25% respectively (Provorov and Tikhonovich, 2003). Upper limits in a nitrogen free situation were higher, at 270 and 550 kg ha^{-1} respectively. These data should be viewed with caution, however, since neither the *O. viciifolia* variety, nor the rhizobia identity were specified.

O. viciifolia was generally reported to fix insufficient nitrogen for its needs and has sometimes shown nitrogen deficiency symptoms in inoculated plants (Burton and Curley, 1968; Sims *et al.*, 1968). The reported insufficient nitrogen fixation of *O. viciifolia* may be associated with energy supply. Sheehy and Popple (1981) found that *O. viciifolia* required gross photosynthesis of 258 kg CH_2O ha^{-1} day^{-1} compared to 234.3 which *Medicago sativa* required. The differences between *O. viciifolia* and *Medicago sativa* in energy requirement may be due to their different leaf area indices (LAI). The LAI of *Medicago sativa* is typically twice that of *O. viciifolia*, and *O. viciifolia* may, therefore,

have less capacity to intercept sunlight and assimilate carbon. This may result in insufficient nitrogen fixation (Sheehy and Popple, 1981). This may explain why *O. viciifolia* has good nodulation activity and a higher nodule weight compared to other legumes.

1.3.5.4. Fertilisation

Nitrate fertilisation is known to reduce nodulation as well as nitrogen fixation of legumes (Hartwig and Nosberger, 1996). Koter (1965) found that low levels of inorganic nitrogen stimulated nitrogen fixation in *O. viciifolia*, but that high levels hindered it. Inoculated *O. viciifolia* with nitrate amendments produced 20 to 30% more forage than inoculated *O. viciifolia* without nitrate (Sims *et al.*, 1968). A yield increase from nitrogen fertiliser was also reported by Meyer (1975). However, Sheehy and McNeill (1988) found that there was no significant difference between the dry matter yield of *O. viciifolia* with or without nitrogen fertiliser application. Badoux (1965) trials with giant sainfoin in Switzerland supported that result. He found that yield was not increased by the application of nitrogen fertiliser; in contrast, there was a 4% reduction after a 90 kg ha^{-1} $year^{-1}$ treatment. These differences in response to nitrogen amendment may be due to difference in soil composition.

There are no specific recommendations for fertilisation of *O. viciifolia* in the United Kingdom. Bland (1971) reported that it responded well to farmyard manure, phosphate and potash but that the optimum amounts of application had not been studied.

In another study, however, Sheehy *et al.* (1984) evaluated the nutrients extracted from soil by *Medicago sativa* and *O. viciifolia* and converted them into fertiliser equivalents. *O. viciifolia* required more P_2O_5 and NO_3 than *Medicago sativa* but less K_2O and $CaCO_3$.

O. viciifolia response to phosphate and potash has seldom been reported. Meyer (1975) found that P_2O_5 and K_2O, either alone or in combination with nitrogen, had very little effect on *O. viciifolia* productivity, recovery or stand persistence. However, Shan *et al.* (1991) found that added P_2O_5 increased *O.*

viciifolia yield. Tufenkci *et al.* (2006) stated that the application of phosphorus, nitrogen and *Rhizobium* inoculum improves yield and nutrient uptake in *O. viciifolia*. The best performances were obtained on inoculated plants with *Rhizobium* species with the application of 40 kg ha^{-1} of nitrogen in addition to 39 kg ha^{-1} of phosphorus. These differences in response to nutrient may be due also to the soil type.

1.3.6. Characteristics and management of the crop for forage and fodder production

O. viciifolia in the United Kingdom was traditionally used mainly as a hay crop, but it could be cut for silage as well (Bland, 1971; Sheldrick *et al.*, 1987). *O. viciifolia* aftermath was used for grazing, and light grazing only in the late autumn was recommended to allow the crop time to replenish root reserves (Sheldrick *et al.*, 1987).

Depending upon growing condition, dry matter yield of *O. viciifolia* may range between 7 and 15 t DM ha^{-1}. Yields are about 20% lower than those of *Medicago sativa*. The contributing factors are mainly: a lower LAI, a less erect canopy structure and a less efficient gaseous nitrogen fixation (Frame *et al.*, 1998). Traditionally cutting normally took place at the bud to mid-flowering stage for the first cut, which can provide about 70% of the total annual yield. Trials in Canada by Goplen *et al.* (1991) showed that regrowth was better if a cut was taken at bud or early flowering stage, but that yield is higher when the first harvest is at a more mature stage. Furthermore, *O. viciifolia* cutting for hay between the 75 and 100% bloom stage can reportedly achieve the best yields and highest yields of nutrients, without appreciable loss of quality, since *O. viciifolia* retains its leaves longer than *Medicago sativa* (Goplen *et al.*, 1991). Protein, lignification and fibre content do not vary significantly between early, medium and late bloom (Mowrey and Matches, 1991).

O. viciifolia regrowth is slow, and it is important to allow enough time to replenish root reserves in order to maintain its persistence and longevity. The behaviour and preference of *O. viciifolia* is similar to that of *Medicago sativa* in

many respects. The recommended interval between cuts for *Medicago sativa* is about 6 weeks, and it uses the root reserve in the first three weeks. In the second three weeks the root reserves are restored to the former level (Aldrich, 1984). Since the regrowth of *O. viciifolia* is slower than *Medicago sativa*, the second and third cuts may be taken at intervals of about 7 weeks after the previous cut. The slow regrowth of *O. viciifolia* compared with *Medicago sativa* may be due to essential differences in the root reserves. The final cut should probably take place when no further regrowth is likely (Mowrey and Matches, 1991; Frame *et al.*, 1998).

De Giorgio *et al.* (2000) compared the root growth with the harvest time. They found that when the crop is cut at 20 centimetres height, subsequent root development was found to be mostly concentrated in the upper layer of the soil, while with a 10 centimetres cut, the root development was much deeper but with small growing intensity.

1.3.7. Seed production

Honey bees (*Apis mellifera*) and leafcutting bees (*Megachile rotundata*) are recommended for pollination of *O. viciifolia* as they are efficient pollinators (Goplen *et al.*, 1991).

Seed production in *O. viciifolia* assisted by bee pollination should be quite successful. For *Medicago sativa*, the pollination success is low because the honey bees learn to remove nectar from the side of the flower to avoid pollen's projection on their head when entering normally from the front. The *O. viciifolia* flower is larger and when tripped will deposit the pollen on the body of the bees. It is assumed that this would not be as irritating for the bees and pollen is therefore collected at almost every visit (Wallace, 1968).

During peak bloom, to optimise the seed yield, Goplen *et al.* (1991) suggested provision of two to three colonies of honey bees or 20,000 leafcutting bees per hectare. For the purpose of this study, honey bees will be used, since they are more easily and economically accessed.

O. viciifolia produces seeds on an inflorescence consisting of 5 to 80

flowers. Each flower has the potential to produce one seed but at best only 55% of the flowers that are pollinated produce seed. This is probably due to the genetic and physiological limitations of the plant (Goplen *et al.*, 1991). A plant may produce 5 to 40 tillers, each having 3 to 5 inflorescences. The number of flowers per inflorescence, inflorescences per tiller and tillers per plant are a function of interrelated environmental and genetic factors (Carleton and Wiesner, 1968).

Several factors are important for seed production. It was noted that seed size increased gradually in the same plant as the number of seeds per head decreased (Carleton and Wiesner, 1968). The optimum seed yield occurs when the plants are cross-pollinated by bees. Seed production of individual plants decreased when competition due to distance between plants increased. Therefore, the plant density determines the performance of the population. Martiniello and Ciola (1994) studied the seed yield components in Italy and found that irrigation improved the seed yield and the components most affected by irrigation are 1000-seed weight, seeds per inflorescence and inflorescence per stem.

O. viciifolia seeds ripen from the base of the flower spike toward the top, and basal seeds shatter from the plants before the upper seeds are ripe. A period of two to three weeks elapses between the opening of the first flower and the withering of the terminal flowers (Goplen *et al.*, 1991). The flowering period starts in early June and lasts about sixty days (Goplen *et al.*, 1991).

The proper stage and harvesting methods of *O. viciifolia* for maximum yield of quality seed is described as follows: seed should be swathed when the seed contains 40% or less moisture and dried in the windrow before threshing (Carleton and Wiesner, 1968). Each hectare should yields at least 500 to 900 kg of clean seeds. Yield up to 1100 kg ha^{-1} have been obtained with some cultivars in Canada (Goplen *et al.*, 1991).

Thomson (1952) reported that unmilled seeds maintain their viability longer than milled seeds. It is therefore important to leave the seeds unmilled if they are going to be stored.

1.3.8. Pest and diseases

O. viciifolia is reported to be relatively free from serious pest and disease problems compared with other legumes (Goplen *et al.*, 1991). *Medicago sativa* suffers from several economically important insect pests such as the *Medicago sativa* weevil (*Hypera postica*) and pea aphids (*Acrythosyphon pisum*), which do not affect *O. viciifolia*. This could encourage the farmers to grow *O. viciifolia* as an alternative solution to *Medicago sativa* (Morrill *et al.*, 1998).

1.3.8.1. Diseases

Several diseases described in the literature occur in *O. viciifolia*. These diseases are quite similar in different part of the world and only a few are really having an economic impact.

1.3.8.1.1. Soil-borne disease

In the United Kingdom, root, crown and stem rot caused by *Sclerotinia trifoliorum* has been reported (Hughes, 1949). Plants infected by this pathogen begin to wither and turn brown during the middle of the growing season (Figure 2). The fungus survives from one growing season to the next in the soil in the form of black sclerotial bodies. Rotation with a non-susceptible crop such as cereals permits a better control of the disease. Crown and root rot caused by *Fusarium* species (mainly *Fusarium solani*) is one of the most important factors affecting longevity in *O. viciifolia* (Mathre, 1968). The symptoms are a dry, brown rot of the inner tissues of the tap root. This fungus may reduce the winter survival of the plants, which are affected. Another soil-borne disease reported in England and Germany is verticillium wilt, caused by the soil-borne fungus *Verticillium albo-atrum*. *Sclerotinia* rot and *Fusarium* crown rot appear to be the most important diseases in temperate climates (Mathre, 1968).

Figure 2: Crown rot on *Medicago sativa* caused by *Sclerotinia trifoliorum*
http://ipm.ncsu.edu/alfalfa/Scouting_Alfalfa/alfalfa_images/fig2.jpg

1.3.8.1.2. Stem and leaf disease

Several stem and leaf diseases has been reported in the United Kingdom and include the following: leaf spot of *O. viciifolia* caused by the fungus *Ramularia onobrychidis* and *Septoria orobina*, ring spot of *O. viciifolia* caused by the fungus *Pleospora herbarum*, the imperfect stage of which is *Stemphyllium botryosum*, leaf and stem spot of *O. viciifolia* caused by the fungus *Aschochyta onobrichidis*, rust caused by *Uromyces onobrychidis*, chocolate spot caused by *Botrytis conerea* and powdery mildew caused by *Erysiphe polygoni* (Mathre, 1968).

Powdery blight (*Aschochyta fabae)* has also been reported on *O. viciifolia* but under different climatic conditions in Iran and Turkey (Eken, 2003).

1.3.8.2. Insects and nematodes

Few insects and nematodes cause damage to *O. viciifolia*. They are described below.

1.3.8.2.1. Root feeding insects

Root feeding insects can make establishment of new stands difficult and

reduce the longevity of an established stand. In this group, weevils of the genus *Sitona* are the most important. *Sitona scissifrons* weevil's adults become active in the field in June. At this time, they crawl on the plants and eat the edges of the leaves, leaving characteristic notches along the leaves. This damage could be disastrous at the seedling stage in the field (Wallace, 1968). Their larvae feed on the roots, and this reduces the persistence of *O. viciifolia* plants because pathogens invade the root scars (Morrill *et al.*, 1998). Other members of *Sitona* (*S. lineata*, *S. calloso* and *S. crinita*) have damaged *O. viciifolia* in Europe (Wallace, 1968). Larvae of a clear-wing moth, *Sesia chalcidiformis* feed also with roots of *O. viciifolia* in Europe (Wallace, 1968).

1.3.8.2.2. Stem and leaf feeding insects

There are a number of other insect species that can damage the stems and leaves of *O. viciifolia* but most of them cause little damage only. Leaf feeding larvae of garden and sugar beet webworm (*Loxostege similalis* and *L. sticticalis* respectively) have been reported to feed on *O. viciifolia* but they appear to be of minor importance (Wallace, 1968). *Medicago sativa* butterfly (*Colias eurytheme*) and closely related species (*C. edusa* and *C. hyale*) are also mentioned as leaf feeding on *O. viciifolia* in Europe (Wallace, 1968). The *Medicago sativa* weevil does not attack *O. viciifolia* but two closely related species, *Phytonomus farinosus* and *Hypera trilineata*, are mentioned as pests of *O. viciifolia* in some European countries (Wallace, 1968). Sucking insects damage the stems, leaves and in some case the developing seeds. One of the most damaging pests is the potato leaf hopper (*Empoasca Fabae*) although it occurs only in localised areas. Lygus (*Lygus elisus*, *Lygus hesperus* and *Adelphocoris lineolatus*) also feed on buds, flowers and seeds (Morrill *et al.*, 1998), but they appear to cause little damage.

1.3.8.2.3. Insects damaging seed production

A number of insect species damage seed production in Europe. The *O. viciifolia* midge (*Contarinia onobrychidis*) is a serious pest in some parts of

Europe particulary in England. The larvae form galls in the flower heads and the seeds fail to develop (Wallace, 1968). *Eurytoma onobrychidis*, the *O. viciifolia* seed chalcid, is also a serious pest in some areas of Europe. The adults lay eggs in the developing seeds and the larvae eat out the inside of the seed (Wallace, 1968). Other insects listed below are also damaging seed production in Europe but are less aggressive: *Perrisia onobrychidis*, *Apion pisi*, *Odontothrips intermedius*, *Otiorhynchus ligustici* and *Meligites erythropus*. Seed production in the USA is decreased by the *O. viciifolia* bruchid, *Bruchidius unicolor* (Wallace, 1968). *Bruchophagous* spp., a seed-infesting insect, has been also reported on *O. viciifolia* in the USA (Morrill *et al.*, 1998).

1.3.8.2.4. Nematodes

Root-knot nematode (*Meloidogyne* spp.) has been found on *O. viciifolia* in the USA (Mathre, 1968). Mathre (1968) reported also that seedlings of *O. viciifolia* are susceptible to the stem and bulb nematode (*Ditylenchus dipsaci*). These nematode species might also damage *O. viciifolia* in Europe but there is no literature report.

1.4. The potential of *Onobrychis viciifolia*

Despite its agronomic problems, *O. viciifolia* has a high potential mainly related to animal nutrition.

1.4.1. Soil improving crop

Sergeeva (1955) reported the superiority of *O. viciifolia* in improving the soil. It is attributed to the many fine ramifications of its abundantly nodulated roots. They die off and thus enrich the soil during the plant's growth period. The fine roots of *Medicago sativa* amount to 4200 kg ha^{-1} in contrast to *O. viciifolia* amount of 16,200 kg ha^{-1}.

1.4.2. Tannin content

Tannins are oligomeric polyphenolic compounds with high molecular weights, which accumulate in plants as natural products of secondary plant metabolism (Caygill and Mueller-Harvey, 1999). Two major classes of tannins are recognised: hydrosable and condensed. Condensed tannins, also called proanthocyanidins, are polymers of flavan-3-ols.

Tannins are often present in the seedcoats of leguminous plants, but they are usually absent from the vegetative part of herbaceous species; there are some exceptions to this, including the members of the *Hedysareae* tribe and some other species such as *Lotus corniculatus* (Bate-Smith, 1973). Scharenberg *et al.* (2007a) found that *O. viciifolia* has a higher condensed tannins content (up to 100g/kg DM) than *Lotus corniculatus*, regardless of the conservation method (fresh, dried or ensiled). There is an even distribution of tannin cells throughout the plants in contrast to other tannin-containing fodder legumes (Skadhauge *et al.*, 1997). It has been shown that the composition of tannins in *O. viciifolia* is highly variable within the cultivar and also for different development stages (Koupaiabyazani *et al.*, 1993a,b). This composition is very complex within the same plant (Marais *et al.*, 2000).

All tannins used to be considered as harmful for mammalian metabolism. Plant phenolics have shown toxic and antinutritional effects in monogastric animals (Lowry *et al.*, 1996). However, there has been a growing interest in tannins due to their positive biological activities in ruminants (Mueller-Harvey, 2006).

Most of the tannin studies to date have been carried out on forage legume species other than *O. viciifolia*, but the results may be useful to understand the positive biological activities in ruminants. McMahon *et al.* (1999) stated that tannins in *Lotus corniculatus* are able to bind the proteins generating 'ruminal escape protein' (Figure 3). This leads to better protein utilisation in the rumen by protecting proteins from early degradation by proteolytic bacteria (Molan *et al.*, 2001). Min *et al.* (2005) also found that condensed tannins from *Lotus corniculatus* inhibited the growth of proteolytic rumen micro-organisms. This

process also leads to environmentally safer forms of excreted nitrogen, lower urinary nitrogen, but slightly higher faecal nitrogen. Barry and McNabb (1999) reported that condensed tannins of *Lotus corniculatus* increased wool growth, milk secretion and reproductive rate in grazing sheep.

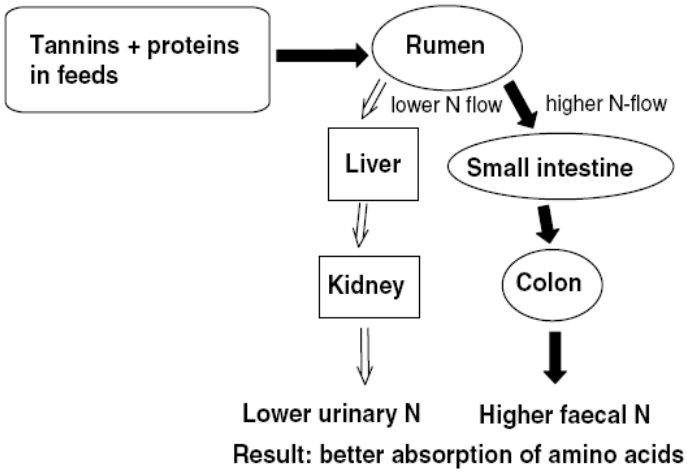

Figure 3: Tannins that bind to dietary protein increase the nitrogen flux from the rumen to the small intestine. This process has been referred to as 'ruminal escape protein'

McMahon et al., 2000

1.4.3. Nutritional properties of *Onobrychis viciifolia*

O. viciifolia possesses important nutritional properties such as high palatibilty and good nutrition value.

1.4.3.1. Palatability

Despite the fact that *O. viciifolia* is a tannin-rich plant, it has always been considered as one of the most palatable forage species. Tannins have been viewed as antinutritional in the past because they can lead to reductions in intake and digestibility of many plants (Kumar and Singh, 1984) but there are interesting exceptions. It has been shown by Parker and Moss (1981) that the voluntary intake by grazing heifers is much higher on *O. viciifolia* than on

Medicago sativa. Scharenberg *et al.* (2007a) found that *O. viciifolia* is more palatable than *Lotus corniculatus* for sheep; it is 20-24% higher than for grasses and 10-29% higher than for *Trifolium pratense* or *Medicago sativa* (Waghorn *et al.*, 1990).

1.4.3.2. Nutritional value

O. viciifolia has good nutritional value; it has a different amino acid composition to *Medicago sativa* and has a high protein quality (Kaldy *et al.*, 1979). In addition, a study by Karnezos *et al.* (1994) showed that the utilisation of the metabolisable energy in *O. viciifolia* is highly efficient. The growth rate in lambs was greater in *O. viciifolia* than with other forages such as *Medicago sativa*. Hoste *et al.* (2005) found also that *O. viciifolia* and *Medicago sativa* have similar effects on milk production by goats.

1.4.3.3. Rumen degradation

Barry and McNabb (1999) reported that the fresh *O. viciifolia* tannins reduce protein degradation in the rumen and this leads to lower ruminal ammonia concentrations. Wang *et al.* (2007) reported also that cattle grazing *Medicago sativa*-*O. viciifolia* mixtures of as little as 85 grams per kg dry matter, had lower ruminal ammonia concentrations than those grazing pure *Medicago sativa* pasture. However, Wang *et al.* (2007) found that, with silage, this reduction of ruminal ammonia concentration occurred only with pure *O. viciifolia* silage. It was concluded that *O. viciifolia* condensed tannins protect protein more efficiently from degradation in grazed than in ensiled material. *Medicago sativa* and *O. viciifolia* can be co-ensiled in order to improve the fermentation and the total tract digestion. The optimal ensiling and ruminal fermentation was obtained with a proportion of 60:40 DM respectively of *Medicago sativa* and *O. viciifolia* (Wang *et al.*, 2007). Scharenberg *et al.* (2007b) showed that in sheep, the condensed tannins from *O. viciifolia* decreased ruminal protein degradation and urine nitrogen losses. The condensed tannins also increased the plasma

concentration of essential amino acids, which indicates that the rumen escape protein is digested in the intestine (Waghorn *et al.*, 1990).

1.4.4. *Onobrychis viciifolia*, a non-bloating forage

Bloat is a digestive disorder occurring in cattle, sheep and other domestic ruminants; it occurs mainly when animals are fed with legume forages. Lush grasses in early spring can also cause bloat. It is caused by the formation of stable proteinaceous foams in the rumen, which prevent gas escape and can often eventually result in animal death (Clarke and Reid, 1974). Thus, it represents a serious problem for livestock producers, who need to limit the quantities of bloating forages (*Trifolium* species and *Medicago sativa* mainly) in the diet of their animals. A two-decade long trial (1973-1993) was conducted to quantify the effect of the forage on the occurrence and the severity of bloat. It was shown that every *Medicago sativa* cultivar tested induced bloat. But *Lotus corniculatus*, cicer milkvetch (*Astragalus cicer* L.) and *O. viciifolia* did not induce bloat. This confirmed the bloat-safe feature of *O. viciifolia* (Majak *et al.*, 1995). As a consequence, *O. viciifolia* can be fed *ad libitum*, grazed or conserved as hay or silage.

1.4.5. Anthelmintic properties

Acquired resistance to anthelmintic medicines has been reported in almost all species of domestic animals (Jabbar *et al.*, 2006). There is therefore a need to find an alternative solution to drugs to control the worm population.

The consumption of tannin-rich plants is suggested to avoid nematode infections. It has been shown for several tannin-rich plants using sheep as a model. In particular, *O. viciifolia* has been shown to have an antiparasitic effect on the most important sheep nematode, *Haemonchus contortus* (Heckendorn *et al.*, 2006). Further studies comparing *O. viciifolia* to other tanniferous forage plants have pointed out *O. viciifolia* to be the most promising candidate for an integrated control strategy against *H. contortus* (Heckendorn *et al.*, 2007). *O.*

viciifolia has also been shown to have a negative effect on nematode egg excretion and a positive effect on host resilience in goats (Hoste *et al.*, 2005; Paolini *et al.*, 2005). The active compounds involved in anthelmintic properties have been determined. Tannins and flavonol glycosides have both been characterised to have an effect on the *in vitro* larval migration of *Haemonchus contortus* (Barrau *et al.*, 2005).

1.4.6. Feed for exotic herbivores in zoological collections

O. viciifolia could be also relevant in zoo herbivore feeding. Some animals resident in zoos, primarily the natural tree browsing feeders such as giraffes, require high quality forage and may suffer sudden and often fatal metabolic problems if this is not provided. High quality conserved *Medicago sativa*, determined as the most suitable artificial forage for many captive browsing herbivores, is often difficult to obtain in temperate climates due to problems encountered in the hay making process. It would be therefore be possible that a forage with a high palatability, like *O. viciifolia*, could be offered as a very beneficial alternative, particularly as this crop can be safely fed on a fresh forage basis without the risk of ruminal bloat occurring as has often been reported to be a problem with the feeding of other legume crops on this fresh fed basis. *O. viciifolia* may also have an anthelmintic effect, which would provide a natural method for controlling gastro-intestinal parasites, this is of particular relevance when dealing with exotic species and the avoidance of excessive pharmaceutical treatments is preferred (Andy Bartley, Zoological Society of London, personal communication).

1.4.7. State of the art on breeding programmes: a potential not fully exploited

Various breeding programmes have successfully improved the agronomic performance of both *Medicago sativa* and *Trifolium* species, but little research has been directed towards improving *O. viciifolia* varieties in Europe. Currently,

O. viciifolia is not widely cultivated due to its inferior agronomic performance compared to *Medicago sativa* or *Trifolium* species.

A few isolated breeders are still registering new synthetic cultivars adapted for specific needs but the breeding programmes are very small and do not take into account the huge diversity available. *O. viciifolia* varieties differ largely in winter-hardiness, maturity, yield potential and many other factors (Shaw, 1968).

Modern bred cultivars of *O. viciifolia* do not rigidly align with one or other of the two main original types, common or giant, but are more flexible in their characteristics. Some well-known landraces are 'Cotswold Common', 'Hampshire Common' and 'Sombourne' for common types and 'Hampshire Giant' and 'English Giant' for giant types. Some new cultivars derived from these two types and Russian landraces are: 'Nova' and 'Melrose' developed in Canada in the 1970's, 'Eski', 'Remont' and 'Remunex' developed in the USA in the 1960's and 1970's, 'Zeus' and 'Vala' from Italy, 'Perly' from Switzerland, 'Fakir' from France and 'Emyr' developed in Hungary (Koivisto and Lane, 2001). Other varieties are still released as the variety 'Shoshone' in 2006 in Wyoming, USA. It has been selected due to good agronomic performances (Gray *et al.*, 2006). A 'G35' *O. viciifolia* was released in New Zealand. Its selection criterion was a better adaptation to New Zealand climatic conditions (Rumball and Claydon, 2005). Some breeding was also carried out in Italy in the last decade (Martiniello, 2005).

In 2010, only 19 varieties of *O. viciifolia* are registered on the European common catalogue (http://ec.europa.eu/food/plant/propagation/catalogues/comcat_agri_2008/37.html). There is no *O. viciiifolia* guideline available for the conduct of tests for distinctness, uniformity and stability produced by the International Union for the Protection of New Varieties of Plants (UPOV). Furthermore, the biological potential of the lines is still not taken into account in the breeding programme.

1.5. A project to understand the value of the sustainable forage *Onobrychis viciifolia*

This Doctor of Philosophy (PhD) programme of work is part of a four year project, called 'HealthyHay'. It is a Marie-Curie training network funded by the European Commission and is comprised of a consortium of 14 partners. This project as a whole evaluates good agronomic, genetic, nutritional and veterinary properties from a unique germplasm collection, and also investigates the unique chemical, nutritional and veterinary properties of *O. viciifolia* and their modes of action (Figure 4). In particular, the beneficial effect of tannins is being investigated. The network will then obtain more information for future *O. viciifolia* breeding programmes by targeting interesting lines.

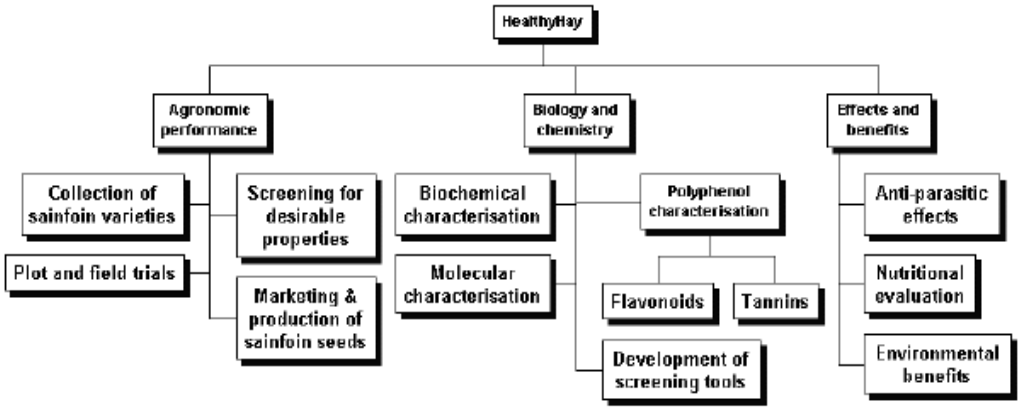

Figure 4: Scientific objectives of 'HealthyHay' project

Research within this project that will be carried out in this PhD

The main purpose of studies for this PhD was to collect germplasm and to characterise its morphological, agronomical, cytological and genetic diversity. To characterise the morphological and agronomical diversity, small-plots were established in field trials. Several field evaluations were done between 2007 and 2010 in order to characterise some morphological traits as well as the agronomic potential of the accessions in order to characterise the phenotypic diversity of the

germplasm. The ploidy and genome size of *O. viciifolia* was also investigated as previous data on these characteristics were sparce and controversial. Some karyotyping studies were also carried out on other *Onobrychis* sp. *O. viciifolia* fingerprinting methods were developed to evaluate the genetic diversity within and between accessions, with the goal of linking traits to genotypes. The taxonomy of *Onobrychis* sp. was also clarified through non-coding DNA sequencing and phylogenetic analyses. This study aimed to assess *O. viciifolia* potential in the context of sustainable agriculture as only scarce and limited information was available on this forage crop. These analyses were designed to constitute a pre-breeding programme, producing data that could be relevant to future breeding programmes. These data must be linked to the analyses performed by Healthy Hay partners in order to select the most beneficial properties that could be associated with improved agronomic potential.

CHAPTER 2. MATERIAL AND METHODS

In this chapter materials and methods used during this study are described.

2.1. Germplasm of *Onobrychis* species gathered

Onobrychis germplasm was collected. It contains mostly *O. viciifolia* accessions. The full germplasm collection is described in Appendix 1.

2.1.1. *Onobrychis viciifolia* germplasm

A germplasm collection of 291 different *O. viciifolia* accessions has been gathered from various sources: nationally and internationally held collections as well as accessions collected from the wild. The majority was obtained from internationally held collections.

The quantity and quality of seed samples supplied were variable. Differences in size, shape, and colour were observed (Figure 5). They were either milled (true seed) or unmilled. Seeds were stored under different conditions, and some of them were more than thirty years old; thus their viability was uncertain. Subsequently, germination rate was often very low (<25%) and varied between 0% and 90%.

Figure 5: Morphological diversity of *O. viciifolia* seeds received

The source of the accessions is described in Table 2.

Table 2: Source of *Onobrychis viciifolia* germplasm used in field trials at NIAB

Number of accessions obtained	**Collection details**	**Country**
162	Germplasm Resources Information Network (GRIN), Washington	USA
27	Institute of Grassland and Environmental Research (IGER), Aberystwyth	UK
24	International Center for Agricultural Research in the Dry Areas (ICARDA)	Syria
16	Gene Bank Research Institute of Crop Production (RICP), Prague	Czech Republic
10	Research Centre for Agrobotany (RCAH)	Hungary
24	Centro de Investigacion y Technologia Agroalimentaria (CITA), Aragon	Spain
2	Cotswold seeds Ltd	UK
3	Caussade semences	France
3	Wild accession collected by Dr Kamalak	Turkey
1	Wild accession collected by Steven Bentley	UK
1	Wild accession collected by Christine Hayot	France
12	Royal Agricultural College (RAC), Cirencester	UK
6	Donated to Dr Irene Mueller Harvey Reading University from various sources	UK

2.1.2. *Onobrychis* species added to the germplasm

Other species of *Onobrychis* were also added to the germplasm collection. Seeds of *O. antasiatica*, *O. atropatana*, *O. hajastana*, *O. michauxii*, *O. petraea*, *O. subacaulis* and *O. transcaucasica* were supplied by the National Academy of Sciences of Armenia. Seeds from *O. radiata*, *O. montana*, *O.*

inermis steven, *O. arenaria*, *O. altissima* and *O. arenaria* were supplied by RICP Prague. *O. antasiatica* accessions were collected from cultivated areas in Armenia, and other species have been collected from the wild. Table 3 summarises the origins of these accessions.

Table 3: Number and sources of *Onobrychis* species accessions other than *O viciifolia*

Number of accessions	**Species**	**Source**
1	*O. arenaria*	RICP: Gene Bank Research Institute of Crop Production in Prague Czech Republic
1	*O. altissima*	NAS: National Academy of Sciences of Armenia
3	*O. antasiatica*	
1	*O. atropatana*	
1	*O. buhseana*	
1	*O. bungei*	
1	*O. cadmea*	
1	*O. cyri*	
1	*O. hajastana*	
1	*O. meschetica*	
1	*O. michauxii*	
1	*O. petraea*	
1	*O. radiata*	
1	*O. subacaulis*	
1	*O. takhtajanii*	
2	*O. transcaucasica*	
1	*O. aequidentata*	IPK: Institute of Plant Genetics and Crop Plant Research in Leibniz Germany
2	*O. altissima*	
4	*O. arenaria*	
1	*O. bobrovii*	

1	*O. caput-galli*	IPK
1	*O. crista-galli*	
1	*O. inermis Steven*	
2	*O. montana*	
1	*O. pallasii*	
1	*O. pulchella*	
2	*O. radiata*	
5	*O. alba subsp. Laconica*	GRIN: Germplasm Resources Information Network in Washington United States
7	*O. arenaria subsp. Sibirica*	
6	*O. biebersteinii*	
5	*O. cyri*	
1	*O. gracilis*	
4	*O. iberica*	
1	*O. argentea*	CITA: Centro de Investigacion y Technologia Agroalimentaria in Aragon Spain
1	*O. pyrenaica*	

These accessions were added to the germplasm because they have interesting biological characteristics, such as extreme temperature tolerance, which might be transferable to *O. viciifolia* in future breeding programmes. Seed shape, size and colour were variable and very different compared to *O. viciifolia* (Figure 6).

Figure 6: Seeds of some *Onobrychis sp.* collected

Seeds from *Lotus corniculatus* and *Lotus pedunculatus* were supplied by Agresearch, New Zealand. There have been several studies on these tannin-rich plants (Hedqvist *et al.*, 2000; Molan *et al.*, 2001) and the comparison between their tannin content and properties was undertaken by other partners within the Healthy Hay Project.

A numerical code starting from 1001 was generated for each accession in order to attribute a simple name to each population; this is an approach generally used in breeding programmes Therefore, these codes will be useful for future breeding programmes. The description of all 360 accessions with their species, variety, code, source, country of origin and status if available is shown on Appendices 1 and 2.

2.1.3. Identifying the source and status of the germplasm accessions

It was necessary to identify the source and status of the accessions in order to explain potential differences observed.

2.1.3.1. Geographical origin

Countries of origin and collection details were sometimes obtained from the passport data provided by the supplier of the seeds. In that case, the US National Geospatial Intelligence Agency (NGA) was used to obtain coordinates. Data tables linking place names of human settlements and geographic features to their latitude and longitude were obtained from the GEOnet Names Server as tab delimited text files (http://earth-info.nga.mil/gns/html/cntry_files.html).

Latitude and longitude data of collection sites were sometimes provided. Accessions with coordinate data were classified with ArcGis 9.0, according to Köppen Climate Zone and Biome. Köppen Climate Zones correspond to the Köppen three character system for categorising climates (Peel *et al.*, 2007) and biomes are described in the International Union for Conservation of Nature and Natural Resources' 'Biotic provinces of the world' (IUCN, 1974), updated by a consortium of conservation organisations (Olson *et al.*, 2001). Despite *O. viciifolia* being cropped artificially, such climatic zones can reflect crucial set of informations that separate different populations.

A full summary of the geographic origins of the accessions is provided in Appendix 2. The accessions' geographic origins (where available) according to Köppen Climate Zones are summarised in Figure 7. Most of the accessions were originally from Europe and Asia (Middle-East) and from temperate regions.

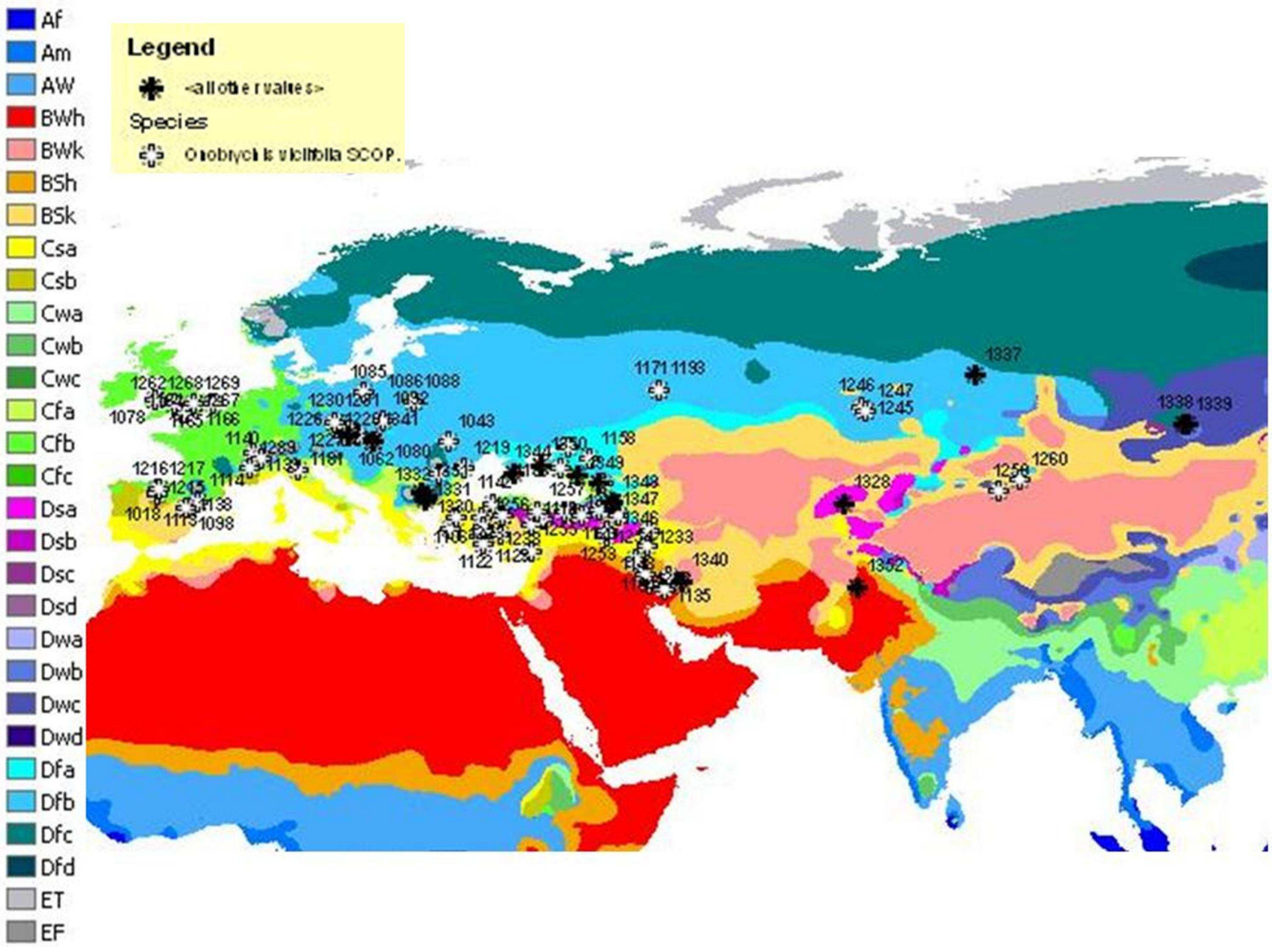

Figure 7: *Onobrychis* germplasm geographic origin, map.produced by ArcGIS 9.0

Köppen climatic zones are represented:

1st	2nd	3rd	Description	Criteria*
A			Tropical	$T_{cold} \geq 18$
	f		- Rainforest	$P_{dry} \geq 60$
	m		- Monsoon	Not (Af) & $P_{dry} \geq 100 - MAP/25$
	w		- Savannah	Not (Af) & $P_{dry} < 100 - MAP/25$
B			Arid	$MAP < 10 \times P_{threshold}$
	W		- Desert	$MAP < 5 \times P_{threshold}$
	S		- Steppe	$MAP \geq 5 \times P_{threshold}$
		h	- Hot	$MAT \geq 18$
		k	- Cold	$MAT < 18$
C			Temperate	$T_{hot} > 10$ & $0 < T_{cold} < 18$
	s		- Dry Summer	$P_{sdry} < 40$ & $P_{sdry} < P_{wwet}/3$
	w		- Dry Winter	$P_{wdry} < P_{swet}/10$
	f		- Without dry season	Not (Cs) or (Cw)
		a	- Hot Summer	$T_{hot} \geq 22$
		b	- Warm Summer	Not (a) & $T_{mon10} \geq 4$
		c	- Cold Summer	Not (a or b) & $1 \leq T_{mon10} < 4$
D			Cold	$T_{hot} > 10$ & $T_{cold} \leq 0$
	s		- Dry Summer	$P_{sdry} < 40$ & $P_{sdry} < P_{wwet}/3$
	w		- Dry Winter	$P_{wdry} < P_{swet}/10$
	f		- Without dry season	Not (Ds) or (Dw)
		a	- Hot Summer	$T_{hot} \geq 22$
		b	- Warm Summer	Not (a) & $T_{mon10} \geq 4$
		c	- Cold Summer	Not (a, b or d)
		d	- Very Cold Winter	Not (a or b) & $T_{cold} < -38$
E			Polar	$T_{hot} < 10$
	T		- Tundra	$T_{hot} > 0$
	F		- Frost	$T_{hot} \leq 0$

- MAP = mean annual precipitation, MAT = mean annual temperature, Thot = temperature of the hottest month, Tcold = temperature of the coldest month, Tmon10 = number of months where the temperature is above 10, Pdry = precipitation of the driest month, Psdry = precipitation of the driest month in summer, Pwdry = precipitation of the driest month in winter, Pswet = precipitation of the wettest month in summer, Pwwet = precipitation of the wettest month in winter, Pthreshold = varies according to the following rules (if 70% of MAP occurs in winter then Pthreshold = 2 X MAT, if 70% of MAP occurs in summer then Pthreshold = 2 X MAT + 28, otherwise Pthreshold = 2 X MAT + 14). Summer (winter) is defined as the warmer (cooler) six month period of ONDFM and AMJIAS

Key from (Peel *et al.*, 2007)

2.1.3.2. Cultivation status

The accessions' cultivation status was sometimes provided. The appellations used by each source were different. The accession status was also sometimes found through a literature search ((Koivisto and Lane, 2001), http://ec.europa.eu/food/plant/propagation/catalogues.html).

It was decided to create three different status designations:

-Cultivar: Registered variety that has been through official examination.

-Cultivated: Traditionally cultivated accession: they can be ecotypes, landraces or breeders line that never became registered cultivars.

-Wild: Accessions collected in wild growing conditions.

2.2. Material and methods specific to the agronomical and morphological characterisation

The methods used for the agronomical and morphological characterisation are described below.

2.2.1. Experimental field used for morphological and agronomical characterisation of the germplasm

A field was used in order to carry out plot trials.

2.2.1.1. Field characteristics

The experimental field was located at the National Institute of Agricultural Botany (NIAB), Cambridge, UK (Figure 8).

Figure 8: Localisation of *Onobrychis viciifolia* field trial: Cambridge, United Kingdom

In the area used for field trials, the soil top layer is a slightly stony clay loam. The subsoil is characterised by a permeable brown slightly stony clay loam becoming grey mottled below 50 centimetres depth. In some places there is stony sandy loam below approximately 70 centimetres. The soil is well drained, but the subsoil is occasionally wet during winter and early spring as a result of fluctuating groundwater. The total area is nearly 0.5 hectare, 66 meters length and 47 meters width. The cropping history of the field is described in Table 4.

Table 4: Previous cropping in the NIAB field used for *O. viciifolia* trial

Year	**Crop**
2002	Set-aside
2003	Sunflowers
2004	Winter barley
2005	Set-aside
2006	Winter wheat
From 2007	Sainfoin

This information was crucial in order to predict potential diseases that might be encountered. The cereal cropping in 2004 and 2006 may have increased the *Fusarium sp.* inoculum potential for subsequent infection of *O. viciifolia*. The set-aside in 2002 and 2005 may have allowed some legumes to grow in the field and therefore increase the potential *Rhizobium sp.* inoculum available for *O. viciifolia* development.

2.2.1.2. Plot organisation

Small-plots were established in the field. There were, when enough plants were available, three replicates of each accession in the field. Each replicate plot was represented by a 1.5 m^2 plot with 36 plants (Figure 9). The space between plots was 0.5 m except for the first replication where the plot rows were spaced by 1.5 m in order to facilitate the seed production.

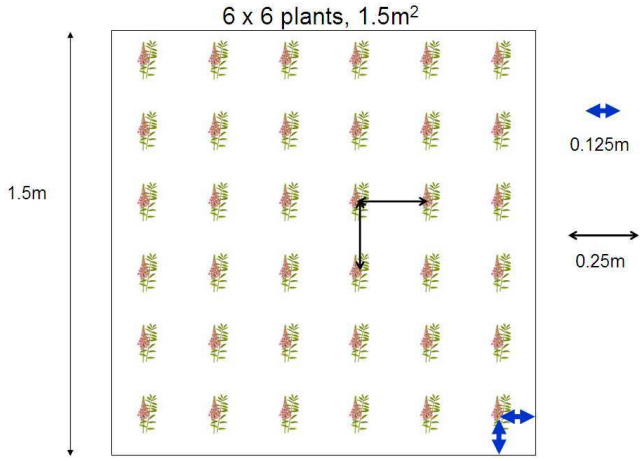

Figure 9: Plot organisation of each accession grown in the experimental field

2.2.1.3. Sowing strategies

Seeds of every accession were sown but only the accessions with sufficient germination rate (>25%) were transferred to the field. This was after being initially raised for two weeks in the greenhouse. After initial plantings, up to 30 percent of the seedlings died in the field. As this strategy had a low success rate and therefore was too time-consuming, it was necessary to improve the methodology at this stage.

In the second phase of planting, the seedlings were grown for longer in the greenhouse (1.5 months instead of 2 weeks) in big permeable pots (Jiffy pots®) (Figure 10) using a *Rhizobium sp.* inoculum (either the UK1 strain isolated from an *O. viciifolia* Cotswold common cultivar or the 6862 USDA strain from the US culture collection) obtained from Legume Technology Ltd, UK. Approximately 5ml of actively growing culture (in specific liquid medium) was sprayed on the bottom of each tray. The plants were then transferred individually to the field as for the previous strategy.

Figure 10: Seedling propagation methods; first phase of planting on the left and second phase of planting on the right

At the end of the planting process, 162 accessions were represented in the field. These accessions are representative of the whole germplasm (Appendix 1).

2.2.2. Harvesting method used for *Onobrychis viciifolia* sample collection

After the agronomic and morphologic evaluations, plots were harvested in 2008 and 2009 in order to supply the other partners of the 'Healthy Hay' project with plant material from the different accessions. Three methods were used to harvest the plants. The plants were cut at 5cm height using shears, string trimmer or with a haldrup (Figure 11). A subsample of each plot was then frozen, freeze dried and sent to the other partners of the project for biological and chemical investigations. Plant material was also supplied for nutritional, environmental

and anti-parasitic studies. Sampling of this plant material was very time-consuming but was crucial to obtain data complementary to the germplasm characterisation.

Figure 11: Harvesting *O vicifolia* foliage in June 2008 using a Haldrup

2.2.3. Pollination method used to produce seeds in 2008

A subset of 75 accessions was selected for seed production in spring-summer 2008 (Appendix 2), in order to renew the seed stocks with recently produced seeds. This selection was made according to several criteria based on the basic evaluation done in January and April 2008. The main criteria were the germination rate of the seeds and the vigour of the seedlings during the initial establishment phase, the general score of the plot and the geographic origin of the accession. Accessions characterised by both vigorous growth and covering a wide range of countries of origin were thus chosen.

The first step required in the process of seed production was to prevent

cross pollination and thus to ensure line purity. For this purpose, each selected plot was covered by a 1.5 m² (1.49/1.49/1.75m) insect-proof tunnel (provided by Diatex, France; Figure 12) before the anthesis (flower opening) stage. These tunnels are made of high density polyethylene with a mesh dimension of 920µm x 920µm, allowing good weather resistance, good air circulation and preventing pollinators from either entering or leaving the tunnel.

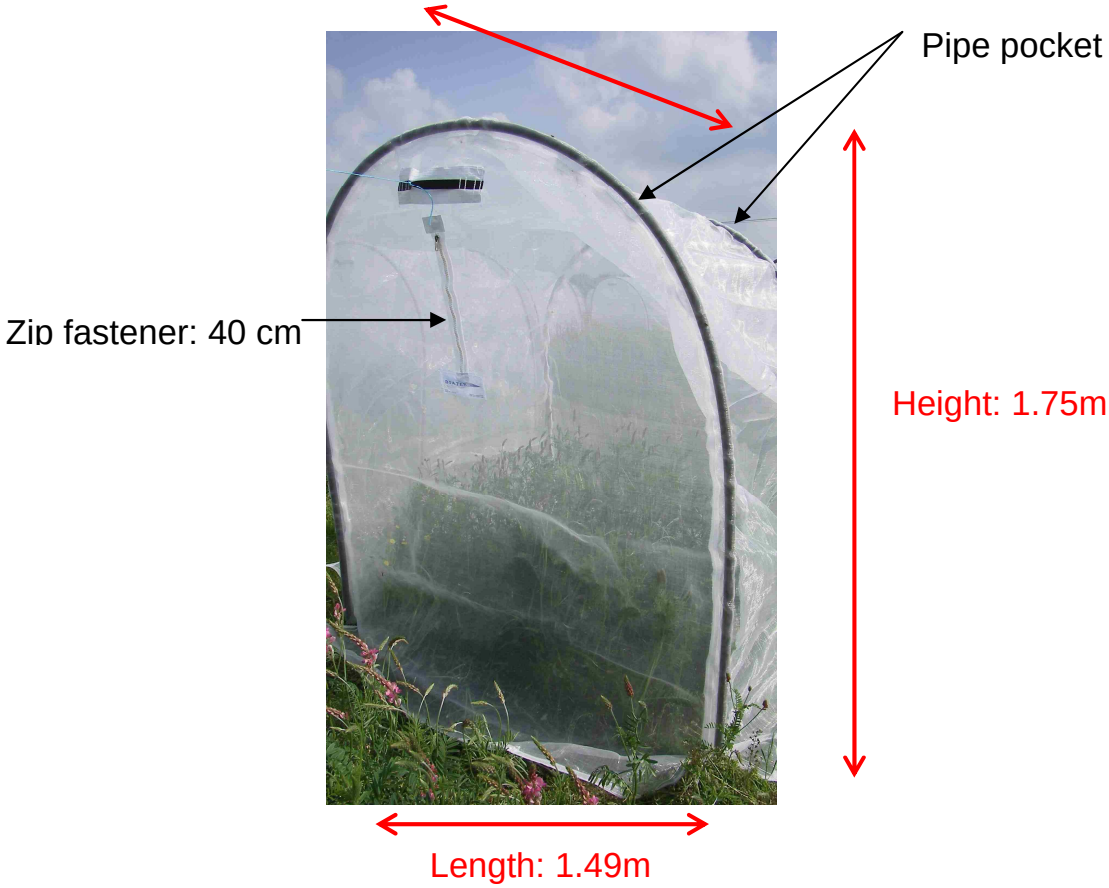

Figure 12: Pollination tunnel used for seed production of *O. viciifolia*

The main pollinators of *O. viciifolia* are a wide range of bee species, including both honey bees and bumble bees. Other insects such as members of the diptera and lepidoptera can also visit the flowers in natural conditions. Commercial plant breeding companies generally use 'mini-hives' containing bees to enable pollination. Mini-hives are comprised of a small bee colony including a queen, workers and larvae contained in a cardboard box. Honeybees are nectar foragers whereas bumblebees are pollen foragers; therefore, the pollination is more efficient with bumblebees. Moreover, honeybees do not work

as efficiently as bumblebees; for example they stop working if it is cold or cloudy. A second consideration is that commercially available minihives of honeybees need to have a larger population to remain viable (circa 100 workers) than bumblebees (fifty workers). Therefore, more management of the hives is necessary for honeybees. Sometimes it may be necessary to kill larvae in order to reduce the number of individuals. Otherwise, as the plot is a confined area, honeybees can 'over pollinate' the plants and this can damage the developing seeds. As a result, bumblebee (*Bombus terrestris dalmatinus*) mini-hives (Biobest, Belgium) were chosen to ensure the pollination in the tunnels. These mini-hives can be used for approximately ten weeks before the population decreases severely. Food supply is available inside the mini-hive (Figure 13).

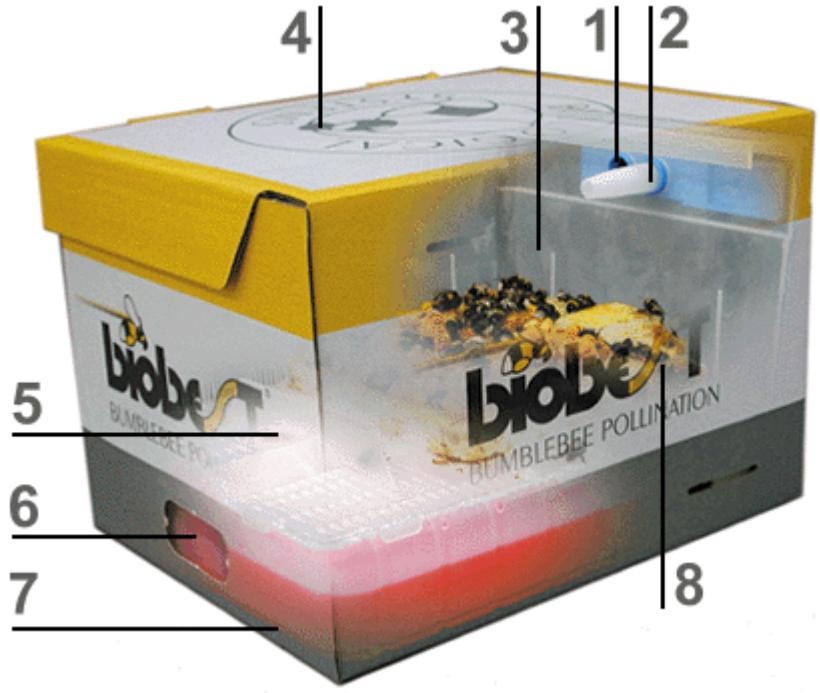

Figure 13: Mini-hive organisation

Key

1. Standard flight opening (in and out)
2. IN only flight opening (not normally used)
3. Transparent inner cover
4. Upper cover, which can be opened
5. Feeding hole through the wick
6. Feeding level, visible from the outside
7. New sugar water formula feed supply
8. The hive compartment with brood

Once anthesis has occurred, a mini-hive was introduced into each tunnel

covered plot to ensure pollination only occurs between plants of a single line. Bumblebees were used in several different plots without risk of cross-pollination as they can clean themselves of pollen. For this seed production, 20 mini-hives were used and kept approximately two weeks in each tunnel. At night, all the bees returned to their hive and the entrances were closed. Then, after 24 hours the bees have cleaned themselves and were moved to another tunnel. During the moving phase, all hives were opened, in order to check the health and the number of bees in each hive.

Seeds were carefully removed by hand and dried in a shaded house. Then they were counted and the viability of 30 of them was evaluated by a tetrazolium test (Roistacher *et al.*, 1953).

2.2.4. Morphologic and agronomic trait characterisation method

In order to rationalise observations and benefit from the experience embodied in some statutory evaluations, UPOV (International Union for the Protection of New Varieties of Plants) reference documents were accessed. On the UPOV website, countries in charge of the "DUS" testing for *O. viciifolia* are recorded. These services are responsible for testing whether the variety is distinct (D) from any other variety and that it is sufficiently uniform (U) and stable (S) in terms of maintaining its characters across years. The examination generates a description of the variety, using its relevant characteristics (e.g. plant height, leaf shape, time of flowering), by which it can be defined as a variety. The countries mentioned are Czech Republic, Germany, Spain, France, Hungary, Poland and Ukraine. Since cultivation of *O viciifolia* has declined in recent years, only one reference document from the German federal authority Bundessortenamt (BSA) was available. These German national guidelines (Table 5) were used in DUS (Distinctness Uniformity and Stability) testing of *O. viciifolia* in the 1990's.

Table 5: German federal authority Bundessortenamt (BSA) DUS testing protocol

for *O. viciifolia*

Trait	Mark	Explanation
Plant height in year of sowing	3, 5, 7	Small, medium, tall
Leaf colour in year of sowing	3, 5, 7	Light green, medium green, dark green
Plant height in spring	3, 5, 7	Small, medium, tall
Leaf colour in spring	3, 5, 7	Light green, medium green, dark green
Time of flowering	3, 5, 7	Early, medium, late
Leaf length	3, 5, 7	Small, medium, long
Leaflet length	3, 5, 7	Small, medium, long
Stem length	3, 5, 7	Small, medium, long
Inflorescence length	3, 5, 7	Small, medium, long
Stem thickness	3, 5, 7	Thin, medium, thick
Plant height at green seed stage	3, 5, 7	Small, medium, tall
Plant colour at green seed stage	3, 5, 7	Light green, medium green, dark green

This DUS protocol was used as an initial basis for development of an *O viciifolia* evaluation protocol within this project. Normally, new varieties are assessed against designated reference varieties, however as no reference varieties were known, the BSA notation based on the 3, 5, 7 numbers had to be adapted.

2.2.4.1. Agronomical characterisation

Agronomical characterisation was performed on 162 accessions from 2007 to 2010. Eleven traits were measured or characterised, sometimes several times in 2008, 2009 and or 2010. They are listed below.

The ‘survival’ of the accession was measured in October 2008 (notation from 1 to 9) and in April 2009 (number of alive plants left). It corresponds to the plant persistency measured either by scoring method (1 for no persistency to 9

with 100% persistency) or by number of alive plants.

The ‘score’ of the accession was measured in April 2008, June 2008, April 2009 and June 2010. It corresponds to the overall state of each plot. Plant vigour, infection by diseases and foliage density were all taken in account to give a representative score (1 for empty plot to 9 for ideal plot with healthy and vigorous plants).

The ‘flowering date’ was measured in 2008 and 2009. It is the date (day of the year) when a plot reached on average the full flowering stage (when 50% of the stems bear inflorescence with opened flowers in the lower half).

The ‘fusarium’ notation was measured in October 2008 and July 2009. It corresponds respectively to the presence (1) or absence (0) of *Fusarium sp.* infection symptoms and to the number of plants showing *Fusarium sp.* infection symptoms per plot.

The ‘mildew’ notation was taken in October 2008. It corresponds to the presence (1) or absence (0) of *Erysiphe trifolii* infection symptoms.

The accession ‘height’ was measured in 2008. It corresponds to the height (in cm) reached on average by full flowering stage plants measured from the soil surface.

The accession ‘weight’ was measured in 2008 and 2009. It corresponds to the dry matter production per plot (in g) in the first cut at full flowering stage estimated by fresh weight converted with dried sample.

The ‘soil cover’ accession abilities were measured in October 2008. It was assessed by a notation from 1 to 9 corresponding to the presence of plant covering the soil (1 for empty plot to 9 for no visible soil).

The ‘flower presence’ was measured in October 2008 and July 2009. It corresponds to the abundance of flowers that regrow after cutting (0 for no flower, 1 for few flowers and 2 for numerous flowers).

The accession ‘regrowth’ abilities were measured in July 2009. It was represented by a 1 to 9 notation and corresponds to the ability of a plant to regrow (1 for no regrowth after cut to 9 for vigourous regrowth).

2.2.4.2. Morphological characterisation

Preliminary morphological characterisation was performed on all accessions in 2008. Then, more complete evaluations were performed on the selected accessions in the second year (2009). Twelve traits were measured or characterised for 3 randomly selected plants in each selected plot, they are listed below.

The ‘leaf colour’ was measured in 2008. It was a 3 to 7 notation (3 for light green, 5 for medium and 7 for dark green).

The ‘flower colour’ was measured in 2008 and 2009. It was a 1 to 9 notation (1 for white flowers and 9 for red flowers).

The ‘stem colour’ was measured in 2008 and 2009. It was a 1 to 9 notation and corresponds to the largest stem colour from totally green (1) to totally red (9).

The ‘number of leaflets’ was measured in 2008 and 2009. It corresponds to the number of leaflets on the reference leaf (first mature leaf at the top of the largest stem).

The ‘leaflet width’ and ‘leaflet length’ were measured in 2008 and 2009. These correspond to the width and length (in cm) of the bottom leaflet on the reference leaf.

The ratio ‘leaflet length/leaflet width’ was calculated in 2008 and 2009.

The ‘inflorescence length’ and the ‘leaf length’ were measured in 2009. They correspond respectively to the measurement (in cm) of the reference inflorescence length (first inflorescence with 1/3 open flowers at the top of the largest stem) and of the reference leaf length.

The ‘habit’ was measured in 2008 and 2009. The notation from 1 to 4 corresponds to the plant growth habit (1: prostrate plant, 2: semi prostrate plant, 3: semi erect plant, 4: erect plant).

The ‘number of leaves per stem’ and the ‘number of stems per plant’ were measured in 2009. They correspond respectively to the number of leaves on the largest stem and to the number of stems on a randomly selected plant.

The ‘stem length’ was measured in 2009. It corresponds to the largest stem length in cm.

The ‘stem thickness’ was measured in 2008 and 2009. It corresponds to the largest stem thickness in mm.

The ‘number of inflorescences per stem’ was measured in 2009. It corresponds to the number of inflorescences on the largest stem.

The accession ‘homogeneity’ was measured in 2008 and 2009. This notation from 1 to 9 realised in full flowering stage (May to June) represented the plot homogeneity in terms of: inflorescence colour, stem colour, foliage colour, growth habit, height, phenological stage, inflorescence length, leaf shape (1 for a perfectly homogeneous plot to 9 for a completely non-homogeneous plot).

2.2.4.3. Selection of accessions of particular interest

A preliminary characterisation was attempted on all accessions in April 2008. As that many accessions represented too much material to analyse for the other partners of the project, a selection of accessions, representative of the diversity (geographic origin, Giant/Common, cultivar/wild) was further characterised (Appendix 1).

2.2.5. Statistical analyses

In order to characterise the accessions several statistical analyses were performed.

2.2.5.1. Basic statistics

Data was compiled in spreadsheets (MS Excel) and basic statistics obtained through GenStat (VSN International).

2.2.5.2 Restricted maximum likelihood

Restricted maximum likelihood (REML) is a method for fitting linear mixed models. In contrast to conventional maximum likelihood estimation, REML can produce unbiased estimates of variance and covariance parameters. Variance components are used in quantitative genetics and plant breeding to assess the relative importance of different sources of variation and in the design of selection programmes. Analysis by REML also provides a simple framework for analyses of data-sets where some data is missing, which was the case here, as it was not possible to evaluate all plots for all traits.

Analysis using REML makes a distinction between fixed effects and random effects. A fixed effect is an experimental treatment of direct interest; as here the accessions' performance. Random effects are generally samples from some real or hypothetical population. In these experiments, "accession" can sometimes be viewed as a fixed effect in order to compare differences between the means of the accessions in our experiment and sometimes as a random effect in order to quantify the causes of variation among *O. viciifolia* plants or plots into genetic and environmental effects. REML analyses were performed in GenStat.

2.3. Cytological characterisation methods

Flow cytometry and microscopy were used for cytological characterisation.

2.3.1. Flow cytometry

Flow cytometry was used in order to characterise ploidy level and DNA content.

2.3.1.1 Ploidy level determination

Ploidy determinations have traditionally been done by counting chromosomes in stained root tips, but this method is laborious, particularly if many plants need to be evaluated. Flow cytometry offers an accurate and rapid method to assess ploidy of either single plants or plant populations (DeLaat *et al.*, 1987).

Flow cytometry analyses of prepared materials were conducted on a flow cytometer partec-Robby. The principle of flow cytometry is explained in Figure 14.

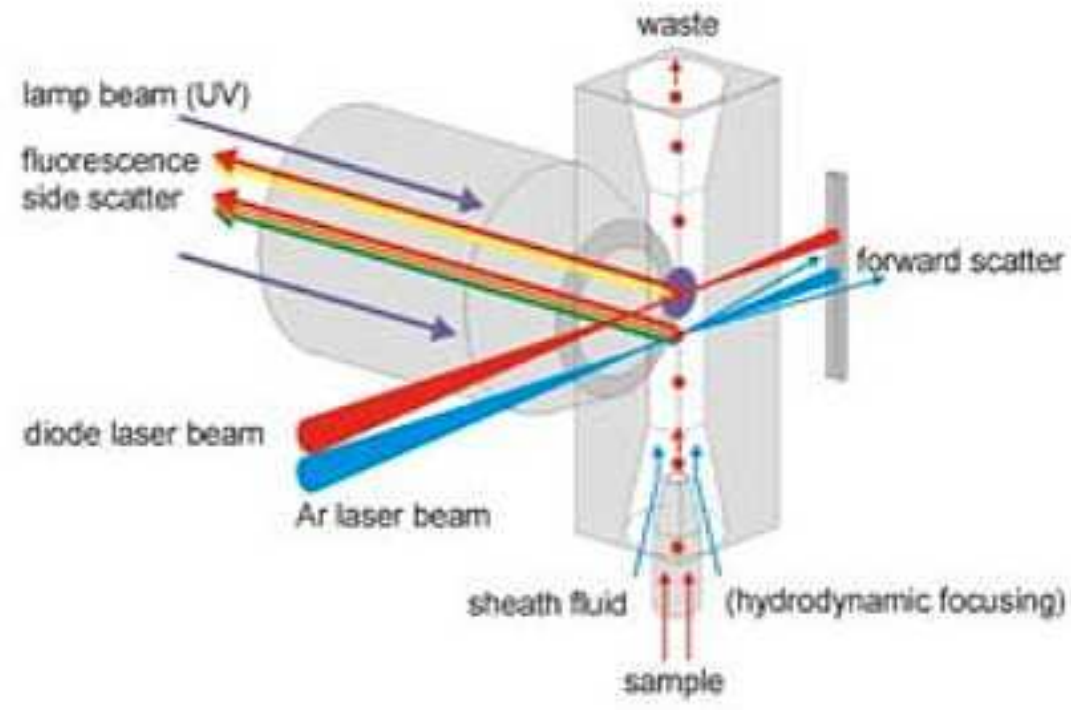

Figure 14: Flow cytometry principle (picture courtesy of Partec GmbH, Germany)

Firstly, the ploidy level stability within an accession was evaluated by comparing the DNA contents of several plants of the same accession.

Some details of specific *O. viciifolia* varieties with known ploidy were found in the literature. *O. viciifolia* accession number 1127 was used,

corresponding to introduction PI 212241 in Kidambi *et al.* (1990a), and known to be tetraploid. Sample preparation method was adapted and the first leaves of young seedlings were used for analysis. 143 *O. viciifolia* accessions were analysed. To determine accession ploidy, a Cystain UV precise P kit from Partec was used.

Small amounts (0.5 cm^2 maximum) of leaf tissue from the tested and the reference sample were chopped together and then separately with nuclei extraction buffer as supplied with the kit for one minute and filtered through a Partec 50µm CellTrics disposable filter. Then, they were stained for two minutes with 4', 6-diamidino-2-phenylindole (DAPI) as supplied with the kit, which binds to the minor groove of DNA and emits in the blue/cyan spectrum. Finally, samples were excited by UV irradiation from a mercury arc lamp and analysed in the blue fluorescence channel (Figure 15).

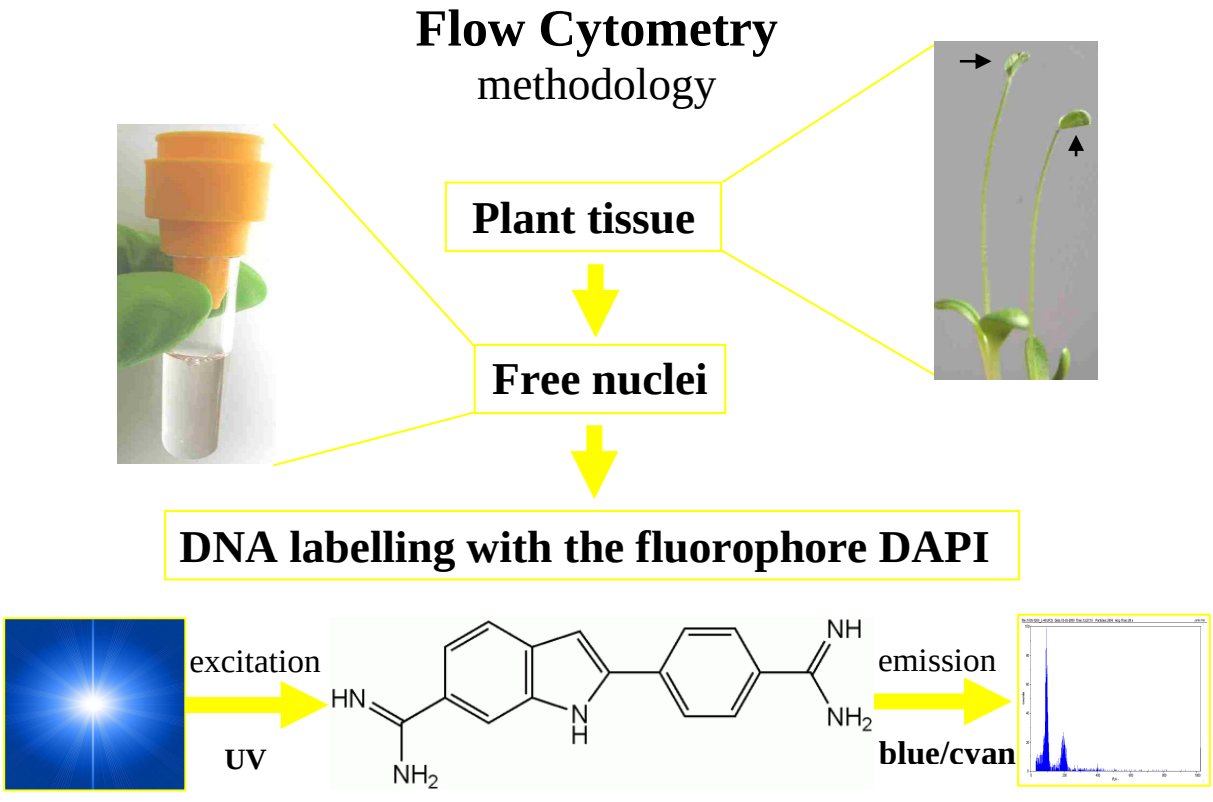

Figure 15: Flow cytometry methodology used in this study

2.3.1.2. Cellular DNA content determination

Flow cytometry procedures were also used to measure the nuclear DNA contents of *O. viciifolia* accessions. The RBG (Royal Botanic Gardens) Kew Plant DNA *C*-values database currently contains data for 5150 different plant species, but no *Onobrychis* species is represented in this database (http://data.kew.org/cvalues/CvalServlet?querytype=1).

To determine the DNA content of *O. viciifolia,* several reference samples of known C-value (Dolezel *et al.,* 2007) were used. *Zea mays* was found to be the most suitable reference sample out of the group provided. Another fluorophore was used as DAPI is GC content dependent and therefore not the best choice considering the difference between species, thus a Cystain PI absolute P kit (Partec) was used. This kit follows a similar procedure to Cystain UV precise P kit, with addition of RNAse and propidium iodide (PI) as the fluorophore. The advantage of PI is that it binds to DNA without significant sequence preference. Professor Dolezel provided a range of plant species with known 2C-value that were used as standards to determine the DNA content of *O. viciifolia* (Dolezel *et al.*, 2007). The best standard was found and the genome size calculated according to the formula: Sample 2C value = Reference 2C value x Sample G1 mean peak position/Reference G1 mean peak position.

2.3.2. Microscopy analyses of root meristematic tissues

To confirm the flow cytometry results, somatic chromosome counts were determined using conventional squashes (of root tip somatic cells, Official seed testing station, Cambridge) visualised under a phase contrast microscope (Axioskop40, Zeiss) equipped with a camera and image software analysis (OpenLab 4.0.2, Improvision). Seeds were placed between moist filter paper at 20°C and left to germinate for 3 days. Young roots were harvested when approximately 1 cm in length, usually after three or four days. The excised roots were immediately placed into a 0.002 M solution of hydroxyquinoline for a period of 4-5 hours at room temperature. The roots were then fixed in Carnoy

solution (3 ethanol: 1 acetic acid) overnight at 5°C. Hydrolysis was then carried out in 1M hydrochloric acid at 60°C for 8-9mins. After hydrolysis, the roots were placed in Feulgen solution (leuco-basic fuchsin) and left for 30mins to stain. Then, the stained meristematic root tips were removed and placed on a slide in a drop of 45% acetic acid and covered with a cover glass.

2.4. Molecular biology methods

Several methods were used to genetically characterise *O. viciifolia*.

2.4.1. DNA extraction

All molecular biology work was performed on DNA so it was necessary to extract good quality DNA.

2.4.1.1. DNA extraction protocols

O. viciifolia foliage contains tannins which, along with many other polyphenols, can dramatically interfere with most DNA extraction procedures and exert negative effects on the DNA quality and yield. To overcome the possible side effects caused by the presence of polyphenols of different types and molecular weight, three protocols for DNA extraction were tested and optimised.

A crude method was modified from Tanksley's DNA micropreparation (Fulton *et al.*, 1995). This protocol is routinely used at NIAB as it gives good DNA extraction from cereals (wheat and barley mainly) and follows these steps (96 wells protocol).

50-100mg of leaf material was harvested and harmful cellular enzymes and chemicals were inactivated with 500µl of buffer (2.5X (25ml) DNA extraction buffer (0.35M sorbitol, 0.1M Tris HCl pH 8.0, 5mM EDTA), 2.5X (25ml) nuclei lysis buffer (0.2M Tris HCl pH 8.0, 0.5M EDTA, 2M NaCl, 2% CTAB), 1X (10ml) 5% Sarkosyl, 0.2g sodium bisulphite and 60µl RNase solution from Qiagen. Samples were ground using a mixer mill for 30s at 30Hz

to allow access to DNA by breaking down the cell wall and membranes. The resulting mixtures were incubated at 65°C for 60mins and shaken halfway through then cooled for 15mins at 4°C. 300µl of chloroform:isoamylalcohol (24:1) were then added in order to purify DNA and the plates were inverted and centrifuged at 1500 x g for 5mins. The supernatant was transfered into a new plate and 340µl cold 100% isopropanol were added to each well to precipitate DNA. The plates were centrifuged at 6000 x g for 5mins and the isopropanol supernatant discarded. 500µl of 70% ethanol were added to the precipitated DNA in order to wash the contaminating salts. The plates were centrifuged at 6000 x g for 5mins and the ethanol discarded two times. Pellets were dried at 65°C for 10mins and re-suspended in 100µl TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.4).

Qiagen Dneasy kit and Amersham Nucleon™ PhytoPure™ Genomic DNA Extraction Kit were also tested. The advantages of using DNA isolation kits over the crude method (described above), is that they are fast, simple, do not contain harmful chemicals such as phenol or chloroform and involve minimal handling. The main disadvantage of kits is their cost, around £150 for 50 reactions.

Qiagen Dneasy Plant kit technology makes use of spin columns, which contain a silica-gel-based membrane that binds the DNA. The DNA, while bound to the membrane, can be washed and cleaned from contaminants and then eluted from the column (membrane) using buffer. The DNA obtained is usually less contaminated than DNA isolated by a crude method. DNA was extracted as per manufacturer instructions.

Amersham Nucleon™ PhytoPure™ Genomic DNA Extraction Kit is based on PhytoPure™ resin, which binds plant polysaccharides. DNA was extracted as per manufacturer instructions.

2.4.1.2 DNA quality assessment

The quantity and quality of DNA extracts were checked by gel electrophoresis. Gels were composed of 2% agarose (w/v) and 0.5X TBE buffer

(0.045M Tris-borate, 0.001M EDTA pH 8). Gels were run at 100V per cm for 30 minutes. DNA samples were mixed with a dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficol (type 400) in water) before loading. Appropriate ladders (10µg/ml) were also loaded to evaluate DNA samples length: Superladder-Low 100bp Ladder and 1kb DNA ladder (ABgene). Gels were analysed by transillumination using a GeneFlash UV imager (Syngene).

DNA was also checked spectrophotometrically using a Spectrophotometer ND-1000 (NanoDrop). The NanoDrop is based on the absorbance measurement at wavelengths 260nm and 280nm. DNA absorbs UV radiation with a peak at 260nm and most proteins absorb UV radiation with a peak at 280nm but both curves slightly overlap between 260 and 280nm. A pure sample of DNA has the 260/280 ratio at 1.8. It is considered that a ratio between 1.7 and 2.0 is relatively free from protein contamination. A DNA preparation that is contaminated with protein will have a 260/280 ratio lower than 1.7.

Finally, PCR (Polymerase Chain Reaction) with two pairs of primers amplifying non-coding regions (an intergenic spacer between *trnT* and the 5' exon of *trnL* and the intergenic spacer between the *trnL* 3' exon and *trnF* tRNA genes) of chloroplast DNA (Taberlet *et al.*, 1991) were performed to check if potential inhibitors had been removed successfully during the extractions and at the same time, to conduct a pilot assessment of the potential genetic polymorphism among different accessions in the collection. The primer sequences are described in Table 6.

Table 6: Primers targeting a non-coding chloroplastic region (Taberlet *et al.*, 1991)

Name Code	**Sequence 5'-3'**	**Expected sizes**
c B49317	CGAAATCGGTAGACGCTACG	300-700bp
d A49855	GGGGATAGAGGGACTTGAAC	
e B49873	GGTTCAAGTCCCTCTATCCC	150-500bp
f A50272	ATTTGAACTGGTGACACGAG	

The quantities used for the PCR were 0.1µl of dNTPs (5mM of each dNTP) (ABgene), 1µl of (10X) buffer including magnesium (Roche), 0.1µl

(0.01U) of *Taq* (*Thermus aquaticus*) polymerase (Fast Start *Taq*, ROCHE), 0.5µl of each primer (200 µM) and distilled water to 10µl. 1µl of DNA extract was used as template.

PCR was performed as: 2mins at 94°C, then 35 cycles of amplification (1min at 94°C, 1min at 50°C, 2mins at 72°C) and 5mins of final elongation at 72°C on an Applied Biosystems Veriti™ 96-Well Thermal Cycler.

PCR products were checked by gel electrophoresis. Gels were composed of 1% agarose (w/v), 10 µg/mL ethidium bromide and 0.5X TBE buffer (45mM Tris-borate, 0.001M EDTA pH 8). They were run at 100V per cm for 45 minutes. PCR products were mixed to a dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 15% Ficol (type 400) in water) before loading. Appropriate ladders (10µg/ml) were also loaded to evaluate DNA samples length: Superladder-Low 100bp Ladder and 1kb DNA ladder (ABgene). Gels were analysed by transillumination using a GeneFlash UV imager (Syngene).

2.4.2. AFLP fingerprinting

A fingerprinting method was needed to characterise the relationship and diversity of *O. viciifolia* accessions. The AFLP fingerprinting method described by Vos *et al.* (1995) appeared to be the best choice as no sequence or marker data were needed to perform it (no such data are available for *O. viciifolia*). An AFLP protocol was developed for *O. viciifolia*. It is described below.

The first step of the AFLP protocol is the adapter preparation. The *EcoR1* adapter was prepared with a concentration of 5 µM. The preparation is described in Table 7. The *MseI* adapter was prepared with a concentration of 50 µM. The preparation is described in Table 8. The *EcoR1* and *MseI* adapter mixtures were heated to 95˚C for 5 minutes. They were then cooled slowly to room temperature and then frozen.

Table 7: *EcoR1* adapter preparation for AFLP protocol

Reagent	**Stock conc.**	**Final conc.**	**Volume (µl)**
EcoR1 adapter 1 CTCGTAGACTGCGTACC	100µM	5µM	25
EcoR1 adapter 2 AATTGGTACGCAGTCTAC	100µM	5µM	25
TE 1mM TrisHCl, 0.1mM EDTA pH 8 at 25˚C	0.1X		450
Total			500

Table 8: *MseI* adapter preparation for AFLP protocol

Reagent	**Stock conc.**	**Final conc.**	**Volume (µl)**
MseI adapter 1 GACGATGAGTCCTGAG	100µM	50µM	250
MseI adapter 2 TACTCAGGACTCAT	100µM	50µM	250
Total			500

Then, it was necessary to estimate the DNA concentration (on a gel and with a nanodrop). The genomic DNA was diluted to 20ng/µl with TE buffer (0.1X 1mM TrisHCl, 0.1mM EDTA pH 8 at 25˚C). Then, the digestion step was performed. The reaction mixture is described in Table 9.

Table 9: AFLP digestion reaction protocol

Reagent	**Stock conc.**	**Final conc./amount**	**Volume (µl)**
Water			14.3
EcoR1 buffer New England BioLabs	10X	1X	2
EcoR1 New England BioLabs	20U/µl	10U	0.5
MseI New England BioLabs	10U/µl	5U	0.5
BSA New England BioLabs	100X	1X	0.2
DNA	20ng/µl	50ng	2.5
Total			20

The digestion mixture was incubated for 1 hour at 37˚C. Then, the ligation reaction was performed. First, the ligation mixture was prepared (Table 10).

Table 10: AFLP ligation mixture preparation protocol

Reagent	**Stock conc.**	**Final conc./amount (in the 25µl)**	**Volume (µl)**
EcoR1 adapter	5µM	0.2µM	1
MseI adapter	50µM	2µM	1
T4 Ligase Buffer New England BioLabs	10X	1X	2.5
T4 Ligase New England BioLabs	400 cohesive end U/µl	200 cohesive end U	0.5
Total			5

5µl of this ligation mixture was added to the 20µl restriction mixture. Then, it was incubated for at least 3 hours at 37˚C. The digested smear was then checked on a 1% agarose (w/v) gel with 0.1µg/ml EtBr and 0.5X TBE buffer (45mM Tris-borate, 0.001M EDTA). 2µl loading buffer (0.25% Bromophenol blue, 0.25% Xylene cyanol ff, 15% Ficol, water) were loaded with 2µl restriction ligation product. The restriction ligation product was diluted 1/10 with 0.1X TE buffer (1mM TrisHCl, 0.1mM EDTA). The pre-amplification PCR was then performed. The pre-amplification mixture is described below in Table 11.

Table 11: AFLP pre-amplification PCR mixture

Reagent	**Stock conc.**	**Final conc./amount**	**Volume (µl)**
Water			36.9
Taq Buffer New England BioLabs	10X	1X	5
dNTPs ABgene	20mM (5mM of each)	0.2mM	0.5
EcoR1-A GACTGCGTACCAATTCA	10µM	0.24µM	1.2
MseI-C GATGAGTCCTGAGTAAC	10µM	0.24µM	1.2
Taq New England BioLabs	5U/µl	1U	0.2
DNA (1/10 dilution of restriction ligation product)			5
Total			50

The PCR programme was: 94˚C for 1min, followed by 20 times, 94˚C for 30s, 56˚C for 1min and 72˚C for 1min. Finally, the programme ended with 72˚C for 5mins concluded by 4˚C.The digested smear was then checked on a 1% agarose (w/v) gel with 0.1µg/ml EtBr and 0.5X TBE buffer (45mM Tris-borate, 0.001M EDTA). 2µl loading buffer (0.25% Bromophenol blue, 0.25% Xylene

cyanol ff, 15% Ficol, water) were loaded with 2µl restriction ligation product. The pre-amplification product was diluted 1/20 with 0.1X TE buffer (1mM TrisHCl, 0.1mM EDTA). Finally, the selective amplification PCR was performed. The *MseI* dNTP mixture was prepared as described in Table 12.

Table 12: AFLP selective amplification *MseI* dNTP mixture

Reagent	**Stock conc.**	**Final conc./amount in 20µl selective amplification**	**Volume (µl)**
Water			6.3
dNTPs ABgene	20mM (5mM of each)	0.2mM	0.2
MseI-C** GACGATGAGTCCTGAGTAAC**	10µM	0.25µM	0.5
Total			7

The *EcoR1* Taq polymerase / buffer mixture was prepared as described in Table 13.

Table 13: AFLP selective amplification *EcoR1* Taq polymerase / buffer mixture

Reagent	**Stock conc.**	**Final conc./amount in 20µl selective amplification**	**Volume (µl)**
Water			7.7
Taq Buffer New England BioLabs	10X	1X	2
EcoR1-A** FAM labelled CTCGTAGACTGCGTACCAATTCA**	10µM	0.1µM	0.2
Taq New England BioLabs	5U/µl	0.5U	0.1
Total			8

Then, 5uL preamplified DNA diluted 1/20, 7µL *MseI* dNTP mixture and 8µL *EcoR1* Taq polymerase/buffer mixture were assembled. The PCR programme was 94˚C for 1min, followed by 13 times 94˚C for 30s, 65˚C (-0.7˚C each cycle touchdown) for 30s and 72˚C for 1min, followed by 23 times 94˚C for 30s, 56˚C for 30s and 72˚C for 1min. Finally it was ended by 72˚C for 5mins and 4˚C.

After the selective amplification PCR, the AFLP fragments were diluted 1/40 (standard) to 1/200 in water, then an aliquot (1 µl) was prepared for examination by diluting with 10 µl HiDi™ formamide and a Liz 500™ oligonucleotide ‘size ladder’ (as supplied by ABI). The formamide was previously fortified with LIZ 500 size ladder in accordance with the manufacturer’s recommendations of 0.5 µl in 10 µl. 1uL of the diluted amplicon was mixed with 9ul HiDi formamide:Liz mixture. An injection time of 30 seconds was used. Amplicons obtained were analysed through an ABI 3730 capillary electrophoresis sequencer.

2.4.3. Simple sequence repeat fingerprinting

Another fingerprinting method that was used was analysis of simple sequence repeat (SSR) fingerprinting (also known as microsatellites) (Blouin *et al.*, 1996). It is based on the detection of various lengths of PCR products, due to the presence of varying numbers of repeats in an SSR in a given genomic region. A variety of primer pairs targeting SSR-containing regions are available for legumes (Table 14). Amplification with these primers was attempted with *O. viciifolia* DNA.

DNA extracts from two different accessions were used as template with a gradient temperature PCR to determine the best annealing temperature for each pair of primers with *O. viciifolia* DNA.

The quantities used for the PCR were 0.1µl of dNTPs (20mM, 5mM of each dNTP) (ABgene), 1µl of (10X) buffer including magnesium (Roche), 0.1µl (0.01U) of *Taq* (*Thermus aquaticus*) polymerase (Fast Start *Taq*, ROCHE), 0.5µl of each primer (200µM) and distilled water to 10µl. It was added to 1µl of DNA extract. A general PCR programme was used for all the primers and a gradient PCR was used to determine the optimal annealing temperature for each primer pair PCR was as follows: 5mins at 95°C, then 40 cycles of amplification (30secs at 95°C, 1min at 48 to 58°C with 2°C gradient, 2mins at 72°C) and 5mins of final elongation at 72°C on an Applied Biosystems Veriti™ 96-Well Thermal Cycler.

A PCR aliquot (1 µl) was prepared for examination on an ABI capillary sequencer by diluting with 10 µl HiDi™ formamide and a Liz 500™ oligonucleotide ‘size ladder’ (as supplied by ABI). The formamide was previously fortified with LIZ 500 size ladder in accordance with the manufacturer’s recommendations of 0.5 µl in 10 µl. 1uL of the diluted amplicon was mixed with 9ul HiDi formamide:Liz mixture.

Amplicons obtained were analysed through an ABI 3730 capillary electrophoresis sequencer. An injection time of 30 secondes was used.

Table 14: Primer pairs targeting SSR regions of legumes

Marker name	Forward primer	Reverse primer	Repetitif motif	Size range (bp)	Reference
1:MtBA01B04R2	CGATCGGAACGAGGACTTTA	CCCCGTTTTTCTTCTCTCCT	(AAG)6	Multiple fragments	
2:MtBB36F05F1	TCCCCTTAAGCTTCACTCTTTTC	CATTGGTGGACGAGGTCTCT	(CTT)5	Multiple fragments	
3:MtBC47B06F1	CCTTTGGTTGATTCAGTTTC	CCAATATGTCACTCCTTGCT	(ATT)7	600	
4:MtBB44F02R1	GGTGATTGGTGTTTTCTGTC	AGCAAAACTATCACCACCAG	(ATG)7	200	(Gutierrez *et al.*, 20
5:BI74	TGTACCAAGCGAATGAAGTGTT	GGGTTGCATCTAACAACAGACA	(TGAG)9	200	
6:AL79	CCCCATTGACGCATTCTTAC	TCCTCAACCAACCACTTCCT	(CTT)8	600	(Zhang *et al.*, 2007)
7:AG81	ATTTTCCAACTCGAATTGACC	TCATCAATCTCGACAAAGAATG	(AG)5	200	(Peakall *et al.*, 1998
8:AW567861	CGCTTCAATGGCTACAATCG	ATCTCCACCAGGGGTTGG	(TCT)7	800	(Zhang *et al.*, 2007)
9:MtBA27D09F1	GAAGAAGAAAAAGAGATAGATCTGTGG	GGCAGGAACAGATCCTTGAA	(AAG)8	Multiple fragments	
10:MtBA04C08R1	TCAACGAGTTCAGCCAGTTC	ATTGCGGCATCTATGGTTTC	(GAA)5	400	
11:MtBB22G10F1	CCAGTGGCAGCTACGGTACTA	GAGACGGAGGAGAAGTTGCTT	(TCC)6	Multiple fragments	(Gutierrez *et al.*, 20

2.4.4. Sequencing analyses of non-coding fragments

Analyses of non-coding DNA sequences were used to clarify *Onobrychis* taxonomy.

2.4.4.1. PCR primers and protocols

In order to clarify the phylogeny of *Onobrychis* species, some regions were chosen for DNA sequencing. In the DNA quality checks, primers c and d gave good quality sequences so the intergenic spacer between *trnT* and the 5' exon of *trnL* was used.

Ahangarian *et al.* (2007) used the ITS (internal transcribed spacer encompassing ITS1, the 5,8S and ITS2) region to study the phylogeny of the closely related Hedysarea so I chose to use also this nuclear region in my study.

Kress *et al.* (2005) reported that *psbA-trnH* is the most variable plastid region in angiosperms. The fragment obtained is very small so this region was chosen as the third region for *Onobrychis* study.

Primers advised by Kress *et al.* (2005) for DNA barcoding of flowering plants were also tried to check which ones could be applied for *Onobrychis* species. All the primers used are listed by pairs in Table 15.

DNA extracts from two different accessions (1197 and 1261) were used to optimise the PCRs.

The quantities used for the PCR were 0.1µl of dNTPs (20mM, 5mM of each dNTP) (ABgene), 1µl of (10X) buffer including magnesium (Roche), 0.1µl (0.01U) of *Taq* (*Thermus aquaticus*) polymerase (Fast Start *Taq*, ROCHE), 0.5µl of each primer (200µM) and distilled water to 10µl. It was added to 1µl of DNA extract. A general PCR programme was used for all the primers and a gradient PCR was used to determine the optimal annealing temperature for each primer pair PCR was performed as follows: 5mins at 95°C, then 40 cycles of amplification (30secs at 95°C, 1min at 48-58°C with 2°C gradient, 2mins at 72°C) and 5mins of final elongation at 72°C on an Applied Biosystems Veriti™ 96-Well Thermal Cycler.

Table 15: Primer pairs used for sequencing

Primers	Sequences	Reference
c B49317	CGAAATCGGTAGACGCTACG	(Taberlet *et al.*, 1991)
d A49855	GGGGATAGAGGGACTTGAAC	(Kress *et al.*, 2005)
psbA-trnHf	GTTATGCATGAACGTAATGCTC	
psbA-trnHr	CGCGCATGGTGGATTCACAATCC	
trnV-atpEf	GTGTAAACGAGTTGCTCTACCA	
trnV-atpEr	CGACATTTGCACATTTAGATGCTAC	
trnC-ycf6f	CCAGTTCAAATCTGGGTGTC	
trnC-ycf6r	CCCAAGCAAGACTTACTATATCC	
ycf6-psbMf	GGATATAGTAAGTCTTGCTTGGG	
ycf6-psbMr	TTCTTGCATTTATTGCTACTGC	
psbM-trnDf	GCGGTAGGAACTAGAATAAATAG	
psbM-trnDr	GGGATTGTAGTTCAATTGGT	
atpB-rbcLf	AGAAGTAGTAGGATTGATTCTCATA	
atpB-rbcLr	GAATCCAACACTTGCTTTAGTCTCT	
rbcLf	ATGTCACCACAAACAGAAAC	
rbcLr	TCGCATGTACCTGCAGTAGC	
ITS5af	CCTTATCATTTAGAGGAAGGAG	
ITS4r	TCCTCCGCTTATTGATATGC	

2.4.4.2. Sequencing of the amplicons

PCR products were treated to remove excess PCR reagents by an EXO-SAP digestion at a rate of 0.07 µl exonuclease I (EXO), 1.0 µl shrimp alkaline phosphatase (SAP), 0.2 µl x10 buffer, 0.73 µl water, and 10 µl PCR product. The SAP dephosphorylates any excess dNTPs and the EXO digests any unincorporated primers. The reactions were incubated at 37°C for 40 minutes and the enzymes subsequently de-activated by heating to 80°C for 15 minutes.

The digested products were used as a template in a second PCR, a sequencing reaction, where fluorescently labelled dideoxyribonucleotide triphosphate (ddNTP) was incorporated into the reaction mixture. Each of the four alternative ddNTPs was

labelled with a fluorophore specific to a nucleotide. When the ddNTPs were incorporated into sequence during sequence extension, the extension was terminated. This modification to PCR has the effect of yielding a series of PCR products of differing lengths, each marked with a fluorophore that signals the nucleotide present at the final point of the extension product. Two parallel sequencing reactions were carried out for each fragment amplified in the initial PCR; one of these reactions included the 'forward' primer from the initial PCR, the second included the 'reverse' primer. The quantities used for the PCR were 1.5µl of (5X) Big-Dye buffer (Applied Biosciences), 0.7µl of Big-Dye (Applied Biosciences), 0.5µl of each primer (200µM) and distilled water to 10µl. It was added to 2µl of ExoSap DNA extract.

PCR was performed as follows: 5mins at 95°C, then 25 cycles of amplification (10 secs at 95°C, 5 secs at 50°C, 4mins at 60°C) and then a decrease at 4°C on an Applied Biosystems Veriti™ 96-Well Thermal Cycler.

The amplicons were purified by ethanol/ EDTA/ acetate precipitation. Two µl of 0.125 M EDTA (pH 8) was added to each reaction followed by 2 µl of 3 M sodium acetate (pH 5.2) and 50µl absolute ethanol. The reaction plate was sealed, the contents mixed and the reaction allowed to incubate in the dark for 15 minutes. The reactions were centrifuged at 3000 x g for 30 minutes, the seal removed and the supernatant liquid removed from the pelleted sequencing products by centrifuging the inverted PCR plate up to 50 x g. The pellet was re-suspended in 70µl 70% (v/v) aqueous ethanol. The reaction plate was sealed, centrifuged at 1650 x g for 15 minutes, the seal removed and the supernatant liquid removed from the pelleted sequencing products by centrifuging the inverted PCR plate at 50 x g for 60 seconds. Any residual ethanol was allowed to evaporate from the plate at ambient temperature in the dark. The BigDye sequencing reaction products were prepared for examination by dissolving them in 10 µl HiDi™ formamide solution (ABI).

Then, the products were run on an ABI 3730 capillary electrophoresis sequencer.

CHAPTER 3. AGRONOMICAL CHARACTERISATION OF AN *ONOBRYCHIS VICIIFOLIA* GERMPLASM

An *O. viciifolia* germplasm comprising 162 accessions (Chapter 2) was assessed during 3 years (2007-2010) for its agronomic potential.

Restricted maximum likelihood (REML) analyses were performed with accessions as fixed terms and spatial localisation of the plots in the field as random, in order to eliminate any possible environmental effect (Chapter 2). Repeating under other field and environmental conditions would have been ideal but could not be realised in the context of this project. These analyses allowed determination of the mean values for various agronomical traits among the accessions, and identified if there were statistically significant differences between the accessions (e.g. that at least one accession was significantly different from the others).

3.1. Diseases and pest characterisation and resistance

A number of diseases and one pest have been observed on the *Onobrychis* accessions growing in the experimental field at NIAB, Cambridge, United Kingdom. The major disease encountered was caused by the *Fusarium sp*. set: *Fusarium solani* and *Fusarium oxysporum* (as confirmed by microscopy observation). Plants infected are dry and the inner tissue of the roots is dark (Figure 16). The disease was characterised by patches of infection in the field. Therefore, the spatial emplacement was taken into account for the analysis of resistant accessions (Chapter 2). Significant differences between accessions were shown for infection notation in October 2008 and July 2009, with p (probability) respectively 0.038 and <0.001, by REML analysis (Chapter 2). Only a few accessions (1177, 1179, 1184, 1185 and 1256), mostly originating from Spain were asymptomatic (Appendix 3) and therefore apparently unaffected by *Fusarium sp* infections. Accessions 1102, 1105, 1114, 1120, 1124 and 1176, mostly from Turkey, were the most severely affected. *Fusarium sp* infections resulted in plant death.

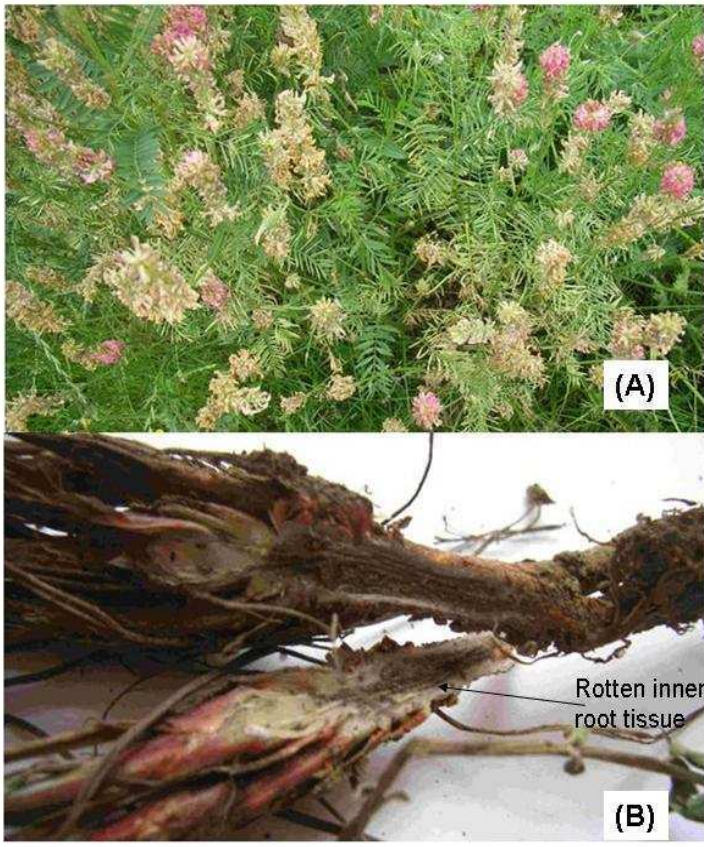

Figure 16: Plant wilting symptoms due to *Fusarium sp*. infection (A) and rotten roots of a *Fusarium* infected plant (B)

A second disease was observed which was characterised by black stems and characteristic pepper spots that were found on the leaves (Figure 17). These symptoms were caused by *Stemphylium sp*. No accession was severely affected by this disease.

A third disease observed was characterised by withered leaves (Figure 18). The infection was due to *Phoma sp.* fungus infection. No accession was severely affected by this disease.

A fourth disease that that was observed only from August to October on a few isolated plants was caused by powdery mildew, *Erysiphe trifolii* (Figure 19). The accession factor was significant ($p < 0.001$), for infection notation in October 2008. Only individuals of a few accessions were affected by *Erysiphe trifolii*. Accessions affected were 1001, 1005, 1013, 1071, 1072, 1125, 1132, 1139, 1165, 1173, 1203,

1206, 1207, 1210, 1250 and 1256. The other accessions were completely free of *Erysiphe trifolii* infection.

Figure 17: Black stems and leaf pepper spots caused by *Stemphylium sp* infection

Figure 18: Symptom of *Phoma sp.* infection on leaves

Figure 19: *Erysiphe trifolii* infected plant showing white powdery spots on the leaves and stems

In autumn, symptoms of adult weevil attack were also observed on many plants (Figure 20). There were no accessions that were severely affected by these attacks.

Figure 20: *Sitona* weevil attack symptoms on leaf characterised by notches on the leaflet edges

3.2. Traits linked with flower production

Full flowering date (Chapter 2) was evaluated in 2008 and 2009. They are presented as ordinal dates. The flowering date distribution is described in Figure 21 below. Significant differences in flowering date were seen (p values of 0.027 in 2008 and <0.001 in 2009). Accessions were in full flowering stage between mid May and mid June in 2008 and 2009.

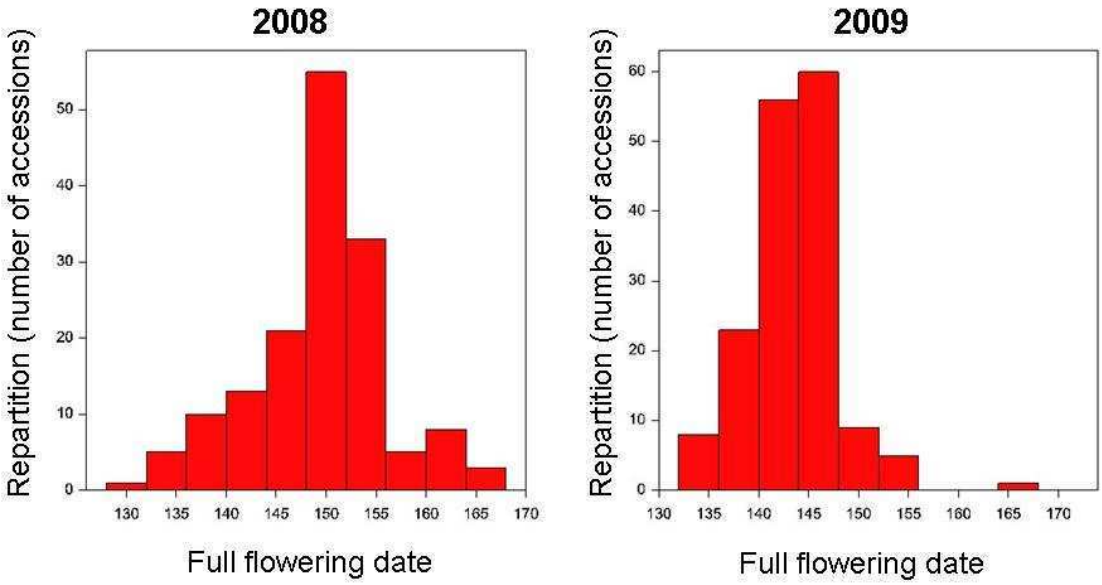

Figure 21: Distribution of full flowering ordinal date in 2008 and 2009

The earliest flowering accessions were 1001, 1111, 1117, 1138, 1139, 1140, 1165, 1187, 1207, and 1210. These accessions, mostly from Western Europe were in full flowering stage before 20th May (corresponding to ordinal dates below 140) in 2008 and 2009.

The latest flowering accessions were 1036, 1072, 1179, 1184, 1185, 1245, 1247, 1249, 1252 and 1256. These accessions, mostly wild types, were in full flowering after 30th May (corresponding to ordinal dates greater than 150) in 2008 and 2009.

The ability of each accession to form flowers again after cutting was assessed in October 2008 (1.5 month after last cut of year 2008) and in July 2009 (1 month after 1st cut of 2009). Significant differences in second flowering were found among

the accessions (p values of 0.004 for October 2008 evaluation and <0.001 for July 2009 evaluation).

Approximately half of the accessions, mostly wild types, had no inflorescence regrowth in October 2008. In July 2009, all the accessions had at least limited inflorescence regrowth (Figure 22).

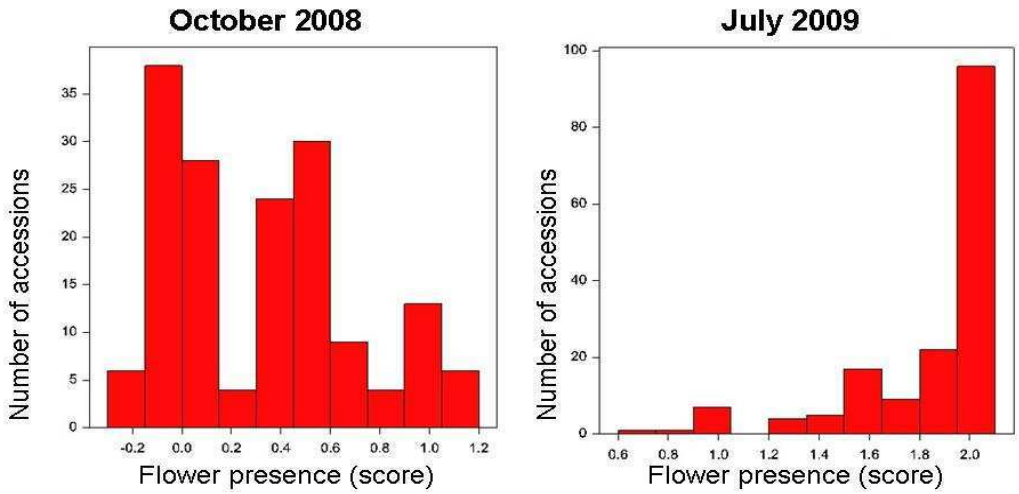

Figure 22: Distribution of flower presence per accession in 2008 and 2009

3.3. Characterisation of traits relating to production

A classic normal distribution was observed with most accessions showing average values, and a few accessions with lower and higher values.

The dry matter (DM) production tended to decrease in the second year, which is probably linked to the fact that some plants did not survive the winter, as shown by the survival measurements (Figure 23). In 2009, only the production of selected accessions (Chapter 2) was assessed due to project duties. According to REML spatial analysis (Chapter 2), accessions were significantly different (p values <0.001). Predicted means are reported in Appendix 3. Accessions 1013, 1156, 1171, 1264 and 1266 performed well in 2008 (above 7000g DM per plot per accession) and 2009 (above 4000g DM per plot per accession). These accessions are mostly registered cultivars (Appendix 1). Some accessions performed very well in 2008 (DM weight production above 7000g per plot) but were not measured in 2009 so

should also be considered. These were accessions 1004, 1009, 1040, 1042, 1044, 1128 and 1168. Some accessions performed very well in 2009 but not in 2008 (compared to other accessions). This was the case for 1019, 1169 and 1197. It was due to difficulty of establishment of these accessions.

Most accessions had an average height at full flowering stage in 2008 between 70 and 110 cm (Figure 24). Significant differences in height were found among accessions (p value <0.001). Some accessions included, on average, plants reaching more than 105 cm from the soil surface (Appendix 3). These accessions were 1044, 1046, 1077, 1104, 1115, 1155, 1201, 1209, 1219, 1248, 1260 and 1266. They are mostly from Eastern Europe (Appendix 2). Some accessions (1016, 1020, 1036, 1139, 1140, 1165, 1179, 1184, 1185 and 1289), in contrast, had a small average height, below 60 cm. They are mostly wild types from Western Europe.

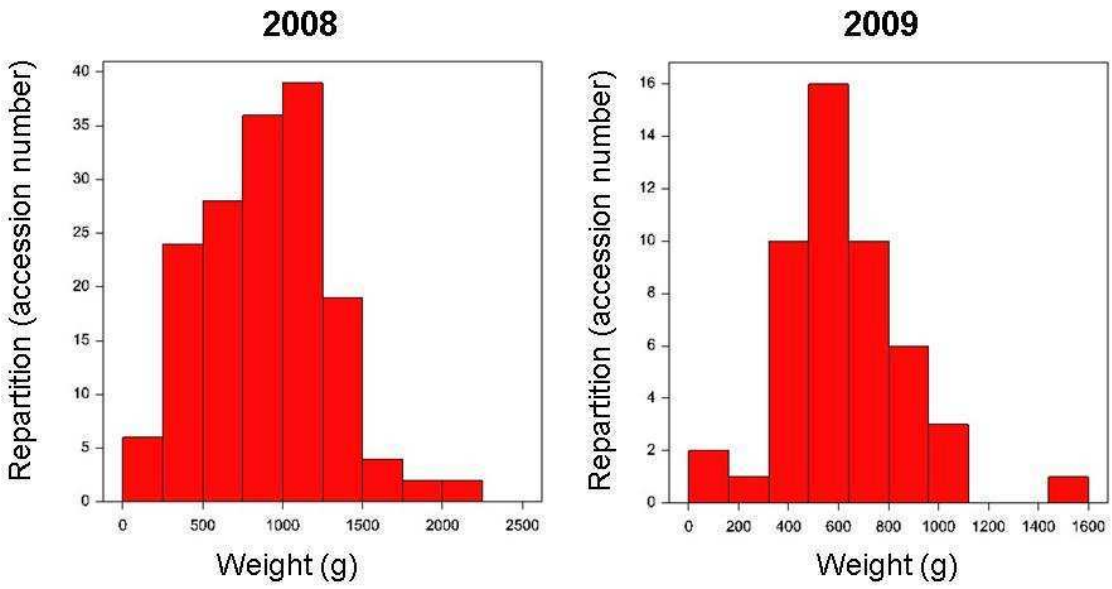

Figure 23: Distribution of dry matter production (in grams) per accession in the first cut at full flowering in year 2008 and 2009

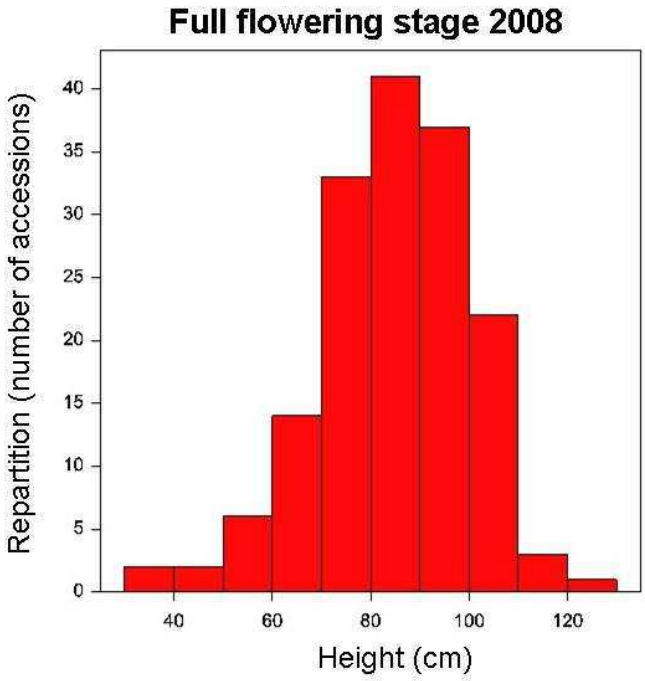

Figure 24: Distribution of average height in centimetres of plants at full flowering stage before first cut in 2008

Each accession's ability to cover the soil in autumn was assessed in October 2008 (Figure 25). Significant differences were seen (p value <0.001). Accessions with a score above 7 were 1001, 1019, 1115, 1165, 1195, 1210 and 1230, mostly cultivars (Appendix 1 and 3). Less well-performing accessions, with scores below 4, were 1009, 1129, 1134, 1184 and 1256.

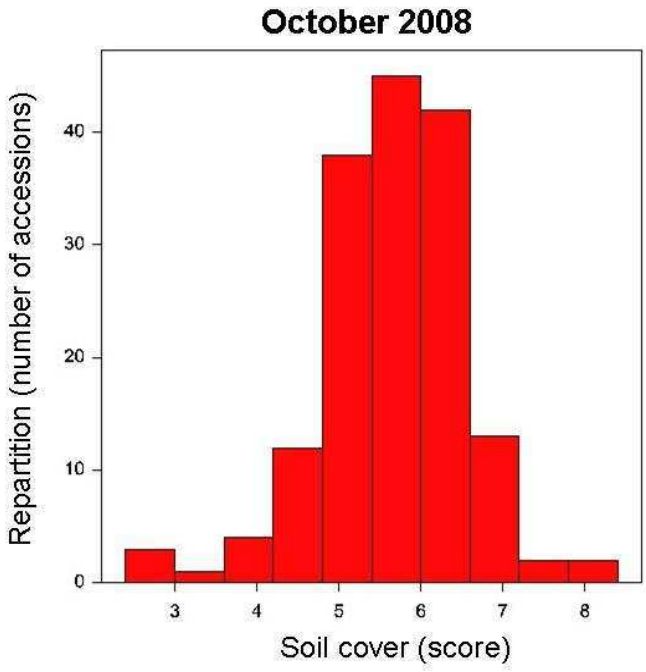

Figure 25: Distribution of soil cover potential (score of 1 for empty plot and 9 for no visible soil) in October 2008

The persistency of each accession was assessed by a survival notation in October 2008 (score) and in April 2009 (number of surviving plants) (Chapter 2). The distribution is described in Figure 26 below. Significant differences in survival were found (p value equal or <0.001). Accessions with best persistency (scores above 7 in October 2008 and counts above 24 in April 2009) were 1001, 1009, 1016, 1019, 1044, 1203, 1213 and 1228 (Appendix 3). Accessions with poor persistency (scores below 4 in October 2008 and counts below 10 in April 2009) were 1102, 1105, 1106, 1108, 1119, 1129 and 1134 (Appendix 3). These are mostly from Turkey (Appendix 2).

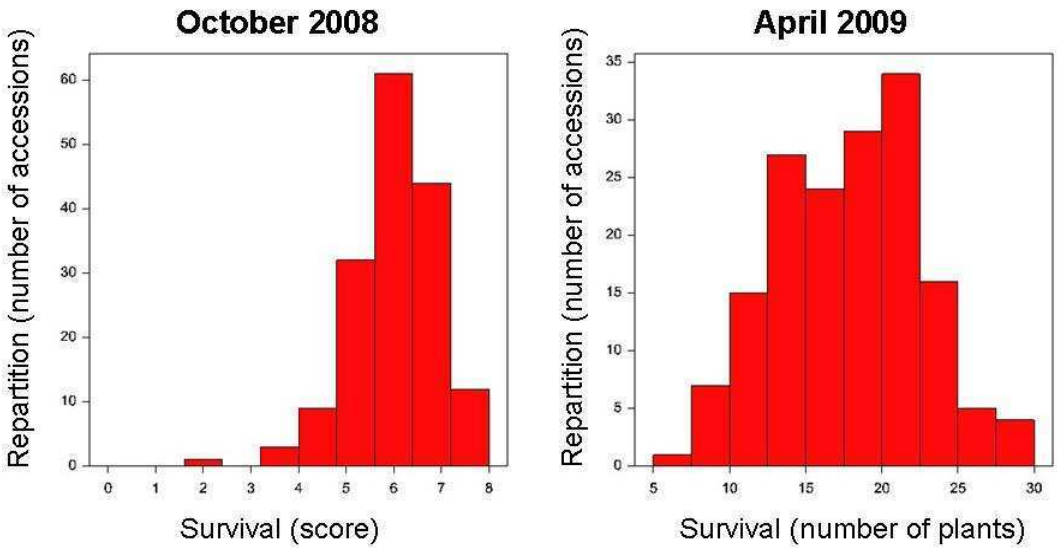

Figure 26: Distribution of survival in October 2008 (score from 1 = no surviving plants to 9 = all plants survived) and in April 2009 (counts of surviving plants)

The plot scores (corresponding to the overall quality of each plot) generally increased in June 2008 compared to April 2008 (Figure 27). A seasonal effect might explain this difference, and the scores were generally relatively high for this first year after sowing in 2007. Significant differences in plot scores were found with p values <0.001 for April and of 0.049 for June. Best performing accessions in 2008 (scores above 8 in April and June of the first production year) were 1007, 1009, 1019, 1040, 1042, 1043, 1044, 1046, 1133, 1145 and 1266 (Appendix 3). These are mostly from Eastern Europe and Asia (Appendix 2).

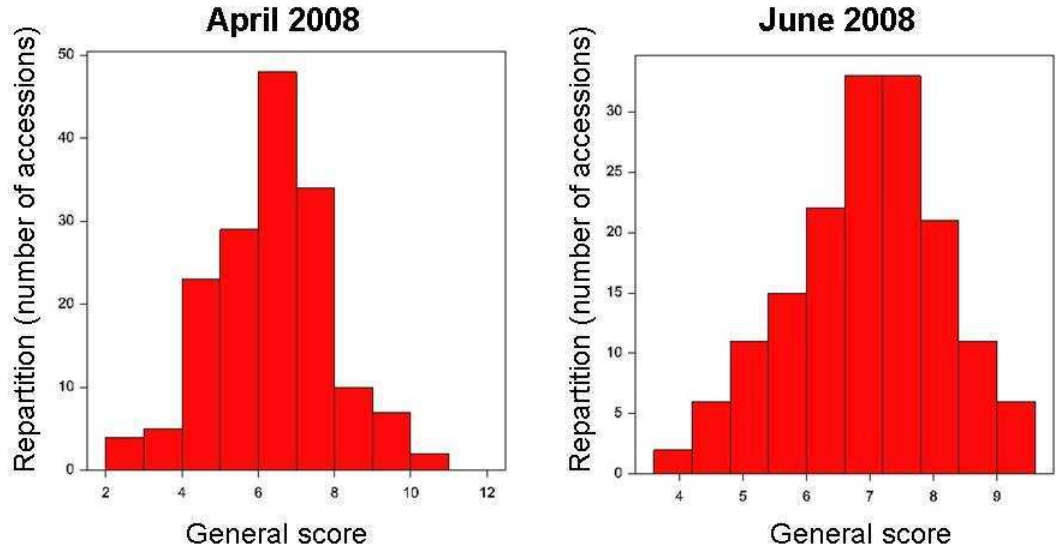

Figure 27: Distribution of overall plot quality scores in April and June 2008

The plot scores generally decreased in 2009 compared to 2008 (Figure 28). This effect was significant with p values <0.001. Best performing accessions in April 2009 were 1019, 1044, 1115, 1173, 1195, 1230 and 1290 (predicted scores above 6).

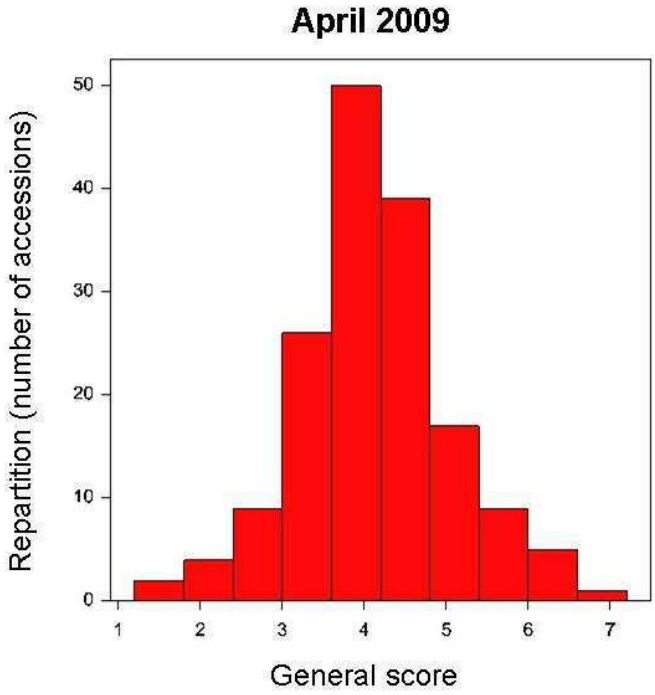

Figure 28: Distribution of overall plot quality scores in April 2009. It corresponds to the overall state of each plot. Plant vigour, infection by diseases and foliage density were all taken in account to give a representative score

The plot scores generally decreased further in 2010 compared to 2009 (Figure 29). Significant differences among accessions were again seen, with p values <0.001. Only 4 accessions had a predicted score above 6 in June 2010. These accessions were 1019, 1044, 1203 and 1289 (Appendix 3).

The accession regrowth ability was assessed in early July 2009 (1 month after the 1st cut in 2009). The distribution of the regrowth ability score is described in Figure 30 below. Significant differences in regrowth ability were seen (p value <0.001). The accessions with the best regrowth abilities (predicted scores above 6) were 1003, 1018, 1026, 1035, 1040, 1041, 1042, 1044, 1115, 1118, 1137, 1148, 1181, 1195, 1213, 1230, 1233 and 1261. Those with poor regrowth abilities (predicted scores below 3) were 1072, 1140, 1179, 1184, 1185, 1256 and 1289, mostly wild types (Appendix 1).

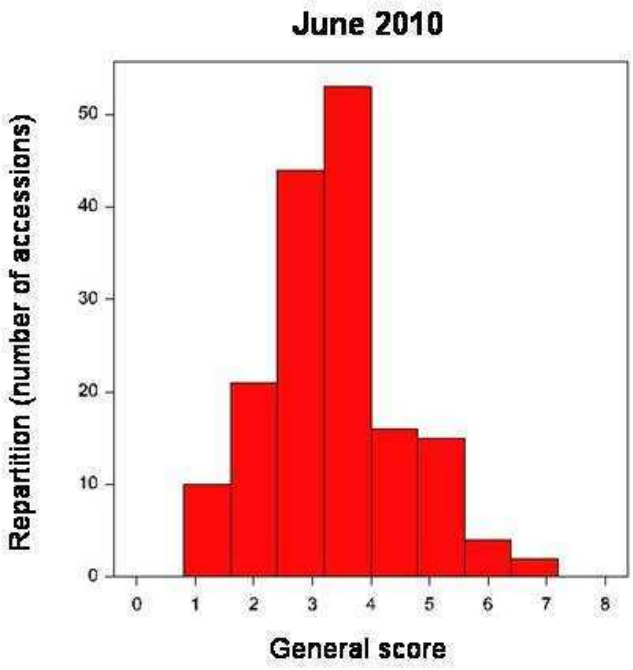

Figure 29: Distribution of overall plot quality scores in June 2010

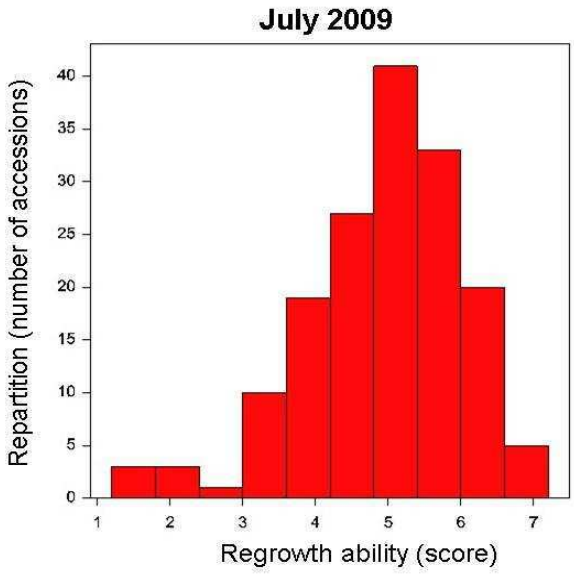

Figure 30: Distribution of regrowth ability score in July 2009

3.4. Characterisation of other traits

Accession homogeneity (Chapter 2) was evaluated in 2008 and 2009 at full flowering stage. It was found to be significant only in 2008. The most homogeneous accessions (score below 1) were 1003, 1012, 1043, 1044, 1077, 1115, 1119, 1121,

1142, 1171, 1172, 1175, 1179, 1199, 1247, 1248, 1264, 1266 and 1292 (Appendix 3).

3.5. Selection of best performing accessions

By summarising the above analyses, it was possible to identify the accessions possessing the best agronomic value. It was shown that the differences observed in a variety of traits were significant. Twelve accessions, which possess the highest values in the key traits, were selected (Table 16).

Table 16: Evaluation of the best accessions (1st best is 1019), relative to best performing accession for each trait, in terms of persistency, soil cover, regrowth, and plot quality scores for 3 production years after sowing. -: <60%, +: 60-75%, ++: 75-90%, +++: 90-100%

Accession	Persistency	Soil cover	Regrowth	Score 08	Score 09	Score 10
1001	+++	++	-	+	++	+
1007	+	++	++	+++	+	-
1009	+++	-	+	+++	+	+
1019	**+++**	**+++**	**++**	**++**	**+++**	**+++**
1040	+	++	+++	++	++	+
1044	+++	++	+++	+++	+++	++
1115	++	+++	+++	++	+++	++
1195	+	++	+++	+	+++	+
1203	++	++	++	++	++	+++
1213	++	++	+++	+	++	+
1230	++	+++	+++	+	+++	+
1266	++	++	+	+++	++	+

Out of these 12 best accessions, only 1 originated from the wild, confirming the agronomic superiority of cultivated/cultivar accessions (Table 17). The majority were from Eastern European countries, and one of them is a cross with another

Onobrychis species (*O. transcaucasica*) (Table 17).

Table 17: Name, geographic origin and cultivation history of the best agronomical accessions

Accession number	**Variety/Code**	**Country**	**Status**
1001	Cotswold Common	UK	Cultivated
1007	Unknown	China	Cultivar
1009	WY-PX-94	Unknown	Cultivar
1019	Taja	Poland	Cultivar
1040	Buceanskij	Romania	Unknown
1044	RCAT028437	Hungary	Unknown
1115	CPI 63755	Turkey	Wild
1195	CPI 63836	Russia	Unknown
1203	Artemovsk	Former Soviet Union	Cultivated
1213	Cross with *O. transcaucasica*	Switzerland	Cultivated
1230	Visnovsky	Czech Republic	Cultivar
1266	Esparcette	Poland	Cultivar

3.6. Effect of geographic and climatic origins

REML analyses were performed to test whether the original geographical origin (Chapter 2), country, climate or biome (Woodward *et al.*, 2004) was a strong determinant of the germplasm performance in the experimental field in Cambridge. Climatic regions were based on the Köppen climate classification (Chapter 2), from climates define by the first (Koppen 1), the second (Koppen 2) and finally the third criteria (Koppen 3) (Peel *et al.*, 2007).

Koppen climate classification (Chapter 2) was shown to affect some traits. Koppen 1, 2 and 3 factors were significant for survival (p value inferior to 0.05).

Koppen 1 and 3 factors affected the plant regrowth and the height (p values inferior to 0.02). Koppen 1 had a significant effect on the date of full flowering in 2008 (p = 0.043) and the flower regrowth in July 2009 (p = 0.01). Koppen 2 had a significant effect on soil cover score (p value = 0.038).

Accessions from alpine polar zones were characterised by an early flowering date, a smaller height and regrowth ability. Accessions from arid and cold zones were characterised by greater height and regrowth ability, whereas accessions from temperate zones were characterised by intermediate values (Table 18).

Table 18: Values of traits significantly correlated to the Koppen 1 climate zone

Koppen1	**Survival Oct 2008 (score)**	**Flowering date 2008 (ordinal date)**	**Flower presence Jul 2009 (score)**	**Height 2008 (cm)**	**Regrowth Jul 2009 (score)**	**Survival Apr 2009 (count)**
Arid	5.4	150	2	89.0	5.2	15
Cold	6.3	152	2	86.7	5.1	19
Polar	6.5	137	1	53.4	2.2	14
Temperate	6.0	148	2	77.4	4.5	17

The country of origin was shown to affect most traits. Country factor was significant for full flowering date in 2008 and 2009 ($p<0.001$) and homogeneity in 2008 (p value = 0.014). Countries also had significant differences in terms of production traits: survival (p = 0.041 for October 2008 and p = 0.03 for April 2009), DM production in 2008 (p = 0.014), regrowth (p = 0.005), height (p value <0.001), general score in 2008 (p value for April 2008 score is 0.008 and is <0.001 for June 2008 score) and soil cover (p value = 0.029).

It appeared that accessions from Eastern European countries (Poland, Romania, Hungary and Czech Republic) generally gave higher values than accessions from all other regions (Table 19). A trend is more difficult to see for lower values; the countries showing the lowest values were only represented by one accession each (Austria and Morocco).

Table 19: Values of traits significantly correlated to the country of origin

Country	Flowering date 2009 (ordinal date)	Score 2009 (score)	Soil cover Oct 2008 (score)	Survival Oct 2008 (score)	Flowering date 2008 (ordinal date)	Height 2008 (cm)	Homogeneity 2008 (score)	Regrowth Jul 2009 (score)	Score Jun 2008 (score)	Survival Apr 2009 (count)	DM production 2008 (g)
Armenia	146	3.8	5.5	6.3	149	88.2	2.7	5.0	6.6	16.8	5423
Austria	147	3.1	4.8	6.0	154	51.5	1.3	4.0	4.7	24.3	1720
Bulgaria	137	4.0	6.4	6.8	137	66.7	1.2	4.0	8.4	13.9	2327
China	147	3.9	5.7	5.8	152	99.3	2.5	5.3	7.8	15.2	5667
Czech Republic	142	5.3	6.5	6.7	147	77.8	2.5	5.9	7.0	22.4	5067
Former USSR	144	4.2	5.8	6.0	150	90.2	2.7	5.1	7.1	17.4	4438
France	136	5.2	5.6	6.3	152	68.9	2.1	4.7	6.4	20.2	3058
Germany	140	5.0	5.9	5.7	142	77.2	5.7	4.6	6.7	19.9	3850
Hungary	144	5.6	6.2	7.6	152	105.0	0.8	6.2	7.9	25.6	7953
Iran	144	4.1	5.3	5.6	151	86.4	2.7	5.8	6.8	16.5	4191
Italy	144	3.8	5.7	6.2	148	91.2	1.8	5.4	8.2	15.9	2923
Kazakhstan	151	3.2	5.2	5.2	164	82.5	1.0	4.0	6.5	16.6	4639
Lithuania	139	3.9	6.1	6.3	138	68.2	2.6	5.3	5.1	20.6	2513
Morocco	142	2.6	4.6	4.5	135	83.3	2.8	4.6	7.6	14.2	4369
Norway	144	4.0	5.8	5.8	153	94.6	2.8	5.0	7.9	21.6	5211
Poland	143	5.0	6.8	6.9	152	92.5	1.7	5.4	8.2	21.4	6203
Romania	147	4.2	5.9	6.6	154	100.8	2.1	5.5	8.1	19.9	6300
Russia	146	4.3	5.8	6.2	154	94.6	1.8	5.2	7.9	19.8	5092

Slovakia	146	5.2	6.3	6.1	*	87.2	2.4	6.3	6.7	22.6	5465
Spain	143	3.7	5.4	5.5	146	70.8	3.4	4.3	6.0	17.0	3776
Switzerland	138	4.4	6.0	6.2	143	74.5	3.2	4.7	6.3	17.1	3718
Turkey	145	3.7	5.1	5.7	151	86.5	2.2	4.7	6.6	15.3	4011
UK	141	4.1	5.9	6.3	145	70.3	3.0	3.9	6.3	18.1	3919
Ukraine	142	4.6	6.1	6.4	150	88.0	2.8	4.9	7.2	19.4	4203
USA	144	3.6	5.6	5.7	151	96.3	2.6	5.4	7.5	14.1	3044

Accessions from regions affected by frost or the absence of a dry season are characterised by better survival and soil cover (Table 20).

Table 20: Values of traits significantly correlated to the Koppen 2 climate zone

Koppen2	**Soil cover Oct 2008 (score)**	**Survival Oct 2008 (score)**	**Survival Apr 2009 (count)**
Significance	**p=0.038**	**p=0.003**	**p=0.007**
Desert	5.4	5.8	19
Dry summer	5.1	5.7	15
Frost	5.7	6.5	14
Steppe	5.3	5.3	15
Without dry season	5.8	6.4	19

Accessions from hot zones have the tallest individuals, those from regions with a cold summer have the shortest individuals with the best survival rates (Table 21).

Table 21: Values of traits significantly correlated to the Koppen 3 climate zone

Koppen3	**Survival Oct 2008 (score)**	**Height 2008 (cm)**	**Regrowth Jul 2009 (score)**	**Survival Apr 2009 (count)**
Significance	**p=0.009**	**p=0.017**	**p=0.015**	**p=0.023**
Cold	5.4	88.3	5.2	15
Cold summer	6.5	52.1	1.7	27
Hot	5.1	93.4	5.6	15
Hot summer	5.8	89.4	5.2	16
Warm summer	6.4	80.2	4.7	19

Biome factor was significant only for height (p value = 0.014). Accessions from regions characterised by grasslands (temperate or montane) were taller than others, those from temperate conifers forest were the smallest (Table 22).

Table 22: Values of traits significantly correlated to the biome zone

Biome	Height 2008 (cm)
Mediterranean forests, woodlands scrub	84.0
Montane grasslands shrublands	100.0
Temperate broadleaf mixed forests	79.7
Temperate broadleaf mixed forests/temperate grasslands, sava	87.4
Temperate conifer forests	67.9
Temperate grasslands, savannas shrublands	92.2

3.7. Comparison of wild and cultivated accessions

REML analyses were use to compare wild and cultivated accessions. Significant differences were found between wild, cultivated and cultivar (Chapter 2) accessions for the overall plot scores in June 2008 and April 2009, soil cover, height and regrowth (Table 23). Wild accessions performed less well for all significant traits (Table 23). Cultivars performed a little less well than cultivated accessions.

Table 23: Means for traits showing significant differences when wild and cultivated accessions are compared

Accession Status	Score Jun 2008 (score)	Soil cover Oct 2008 (score)	Regrowth Jul 2009 (score)	Score Apr 2009 (score)
Cultivar	7.1	5.9	5.2	4.5
Cultivated	7	5.7	5.1	4.1
Wild	6.2	5.3	4.4	3.8
Probability value	0.004	0.012	0.003	0.006

3.8. Seed production

The seed produced were evaluated in terms of quantity (number of seeds obtained and seed weight) and viability (tetrazolium test on 30 seeds for each accession). A positive tetrazolium test should result in red coloured seeds. Non-viable seeds are partly or entirely white/green (Figure 31)

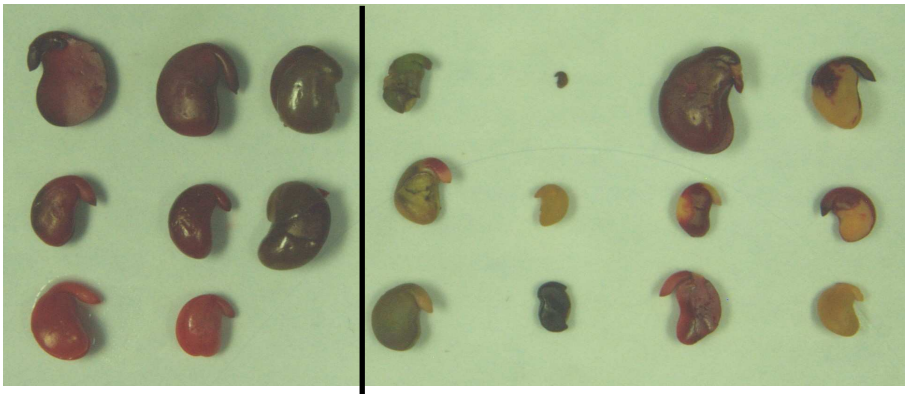

Figure 31: Examples of tetrazolium viability test results obtained with *O. viciifolia* seeds. Seeds totally red are positive (a); negatives are partly or entirely non-red coloured (b)

Although large numbers of seeds were generally obtained, 15 accessions produced less than 1000 seeds and 25 accessions produced less than 70 % viable seeds. Only 8 accessions produced between 90 and 100% viable seeds. Large differences in seed weights were observed (Table 24).

Table 24: Number of seeds, 15 seed weight and viability of seeds produced per accession

Accession	**Number of seeds**	**Weight of 15 seeds (g)**	**% viability (tetrazolium test)**	**Accession**	**Number of seeds**	**Weight of 15 seeds (g)**	**% viability (tetrazolium test)**
1001	2013	0.41	76.7	**1132**	1494	0.46	86.7
1003	6381	0.45	93.3	**1133**	439	0.37	66.7
1005	6198	0.38	76.7	**1134**	558	0.32	56.7
1007	1747	0.43	83.3	**1141**	621	0.46	70.0
1008	715	0.32	80.0	**1142**	2555	0.35	90.0

1013	639	0.51	80.0	**1145**	615	0.37	70.0
1018	2070	0.29	53.3	**1155**	11656	0.28	60.0
1019	6956	0.35	60.0	**1156**	1591	0.37	86.7
1040	4623	0.4	83.3	**1163**	5141	0.43	80.0
1042	577	0.37	86.7	**1164**	1742	0.4	76.7
1043	9380	0.33	86.7	**1169**	1002	0.33	46.7
1044	12064	0.36	100.0	**1170**	1196	0.4	66.7
1045	2322	0.44	86.7	**1171**	3440	0.3	56.7
1046	17837	0.31	76.7	**1187**	4855	0.44	80.0
1071	2628	0.47	70.0	**1188**	786	0.36	83.3
1077	8253	0.34	86.7	**1189**	2500	0.43	63.3
1100	3478	0.37	70.0	**1190**	7944	0.3	43.3
1102	2978	0.34	66.7	**1196**	8414	0.37	83.3
1103	12971	0.37	66.7	**1197**	6904	0.39	63.3
1104	12732	0.44	76.7	**1198**	2588	0.46	80.0
1105	3408	0.44	60.0	**1199**	2227	0.39	80.0
1106	3368	0.48	66.7	**1200**	2160	0.43	80.0
1108	1420	0.43	83.3	**1201**	4918	0.37	76.7
1110	1859	0.3	43.3	**1202**	5584	0.33	86.7
1111	2351	0.48	90.0	**1204**	882	0.41	86.7
1112	3656	0.35	33.3	**1205**	2369	0.33	63.3
1113	1199	0.42	86.7	**1207**	883	0.41	56.7
1114	5907	0.34	70.0	**1209**	700	0.41	83.3
1115	1953	0.4	56.7	**1210**	3215	0.34	53.3
1116	1574	0.32	93.3	**1211**	2299	0.45	80.0
1117	373	0.27	83.3	**1212**	2153	0.22	13.3
1118	4942	0.43	70.0	**1213**	2773	0.45	100.0
1119	3898	0.42	43.3	**1214**	1188	0.32	70.0
1120	3867	0.42	70.0	**1218**	1060	0.41	73.3
1121	2018	0.48	86.7	**1220**	4514	0.4	93.3
1123	17	0.37	Unknown	**1230**	1944	0.37	73.3
1127	547	0.35	90.0	**1260**	694	0.4	63.3
1128	1434	0.25	53.3				

3.9. Discussion

Agronomic characterisation of *O. viciifolia* was a challenging task. A system for evaluation of the different varieties had to be defined since an accepted system was not available (Chapter 2). There were very few details in the literature on which to base a new system. The characterisation was found to be time consuming, considering the number of accessions to be evaluated. There was a need, therefore, for a rapid and coherent characterisation approach. A system for fast and repeatable scoring of traits was needed for effective evaluation, replacing actual measurements for most traits. The inter-plot variability for some accessions was also an important factor driving the need for a scoring approach to overcome this variability.

This agronomic characterisation was important to find trends and differences between the germplasm accessions. This system would not work in a rigorous plant breeding programme, which would have required a different experimental design. However it is effective in the production of a valuable database to select accessions of greater agronomic interest among the germplasm prior to embarking on a breeding programme.

For all traits considered, considerable diversity was observed in the germplasm collection. This diversity was expected given the wide range of geographic origins and cultivation status of the germplasm. Most of these traits were shown to be significantly different through REML analyses. Indeed, this diversity in different agronomic traits represents an interesting source for future traditional breeding programmes. It undoubtedly reflects the impact of climate, landscape, agricultural use and history on the accession phenotypes. A similar agronomic evaluation performed by Negri *et al.* (1987) on a limited germplasm from central Italy has also shown considerable diversity in traits. Furthermore significant diversity in dry matter weight was also observed in a cultivar screening study (Hwang *et al.*, 1992).

Several pests and diseases have affected the accessions. However, it appears that *O. viciifolia* is generally resistant to diseases (Chapter 1). The main problem that was encountered at the site in Cambridge was *Fusarium solani* and *Fusarium oxysporum* infection, which can spread significantly in the field. A previous study

found no significant resistance to *Fusarium* in *O. viciifolia*, except the Nova cultivar, which was less affected (Hwang *et al.*, 1992). In this study, the Nova cultivar was affected in similar proportions to other accessions. A few accessions were found to be unaffected by *Fusarium sp.* and thus represent a potential source for breeding. This resistance will need to be confirmed by further rigorous field pathology to determine the susceptibility of selected lines, and the potential resistance mechanism should be studied to see if it is possible to transfer this into new cultivars.

Conversely, only a few accessions were found to be significantly affected by *Erysiphe trifolii*. Given the very humid conditions in England, which are known to favour powdery mildew development, it can be considered as a very minor threat to *O. viciifolia*.

Other diseases appeared to have marginal effects that plants can generally survive. Attacks from weevils also affected the plants only marginally, with predation by adults recorded. The larvae, which feed on the roots and can cause significant damage to them, were not observed.

The normal distribution of traits clearly indicates that *O. viciifolia* represents a coherent agronomic group with only a few extreme accessions for each trait. In most cases the binomial distinctions erect/prostrate and Giant/Common (Thomson, 1951a) cannot be clearly applied. The Giant/Common distinction was clearly observed only for a few accessions that were defined accordingly. Most accessions appeared to have mixed Common and Giant characteristics and did not strictly adhere to either of these agronomical definitions. This has also been observed for locally adapted *O. viciifolia* landraces from the south-east of France (Prosperi *et al.*, 1994). Similarly, most accessions displayed habits that were between prostrate and erect. The most likely explanation is that local agricultural use histories have in many cases buffered and amalgamated the major differences that may be observed among *O. viciifolia* landraces.

From the various traits measured, some accessions have been highlighted as having greater agronomic potential. Somewhat surprisingly, the majority of these agronomically superior accessions originated from Eastern European countries. This tends to confirm that modern breeding effort on *O. viciifolia* has not been intense and that agricultural selection has been so far the only method that has been used to improve its agronomic properties. It indicates again that the potential for *O. viciifolia*

agronomical improvement is very important and that locally selected and adapted accessions will be a crucial starting point for a modern breeding effort.

Agronomic traits were shown to be strongly determined by the geographic (and associated climate) of the accessions. A general distinction was again found between accessions from Eastern European countries and accessions from the rest of the world. Generally, Eastern European accessions performed better in terms of biomass, soil cover and general state than the others. It reinforces the evidence that a lot of these Eastern European accessions have high agronomic potential. Accessions coming from a polar/mountainous climate had an earlier flowering date and a shorter lifecycle than the others with very slow regrowth. Thus, their intrinsic adaptation to their local climate was not disturbed by growth under a different climate. In contrast, accessions from dry climates which normally experience longer summers (arid and cold) were characterised by longer lifecycles. It is apparent that some intrinsic properties in *O. viciifolia* are strongly connected to the climate and should be considered in breeding programmes.

The cultivation status was found to significantly affect certain important agronomic traits (regrowth, plot score and soil cover). Cultivars and cultivated accessions were superior for all these traits as compared to wild accessions. A slight superiority was also found for cultivars compared to cultivated accessions. Such differences are not surprising and probably show that several of the improved agronomic traits of cultivated lines as compared to the wild *O. viciifolia* accessions have resulted from slow improvements to produce local landraces for agriculture use. Therefore, any further beneficial traits found in wild *O. viciifolia* (or other *Onobrychis* species) such as biochemical properties, nutritional properties and environmentally friendly properties will have to be transferred to the best agronomically improved lines.

Seed production was generally satisfactory though some accessions produced a reduced number of seeds. This may have been partly due to reduction in the bumblebee numbers or activity as the mini-hives were used several times (Chapter 2). The seed viability was generally quite low which tends to predict that germination rates will not be satisfactory. This probably indicates a problem in the seed production process and led to the seed production not being reattempted. Diversity in

seeds weight was observed, which was expected given the overall agronomical traits diversity and the fact that seeds volume has been a recurrent issue in *O. viciifolia* agricultural seeds production (Cash and Ditterline, 1996).

CHAPTER 4. MORPHOLOGICAL CHARACTERISATION OF *O. VICIIFOLIA* GERMPLASM

A morphological characterisation was performed during the *O. viciifolia* field trials (Chapter 2).

Restricted maximum likelihood (REML) analyses were performed with accession as fixed terms and plot replication and plant replication in the plot as random, in order to take into account any possible environmental effect (Chapter 2). REML analyses were used to determine the mean values of various morphological traits among the accessions, and to identify if there were statistically significant differences between the accessions (e.g. that at least one accession was significantly different from the others).

4.1. Preliminary morphological characterisation of germplasm

A preliminary morphological characterisation of *O. viciifolia* germplasm took place in spring and summer 2008. Considerable diversity was observed in most of the evaluated traits: length, thickness, number and colour either for stems, leaves (Figure 32), leaflets or flowers (Figure 33) and habit (Figure 34). Temporal and methodological problems arose as the time frame to do the measurement was short and the number of measurements was important. This was complicated by the need to increase the number of measurements due to the huge diversity within an accession. As a result, it was decided to focus the in depth morphological characterisation on the accessions that were selected for the more thorough analyses (Section 2.2.4.3.).

Figure 32: Leaf diversity (shape, color) observed in 2008

Figure 33: Flower colour diversity (ranging from white to purple) observed in 2008

Figure 34: Erect growth habit plant displayed by a plant from accession 1005 (left picture) and prostrate growth habit of a plant from accession 1179 (right picture)

4.2. Morphological trait characterisation

Several morphological traits were characterised in *O. viciifolia*. These traits were shape, number and colour of the leaves, stems, inflorescences and whole plant (Chapter 2). Traits were either measured directly or via a scoring method (Chapter 2).

4.2.1. Traits linked with number and dimension

In 2008, the growth habits of all accessions were assessed by the plot majority. In 2009, the growth habit of three plants within each selected plot was assessed. Significant differences in the growth habit were observed between accessions (p values below 0.001 in 2008 and 2009). Most accessions had erect or semi-erect growth habit (score between 3 and 4). Only a few accessions showed prostrate or semi-prostrate growth habit (score below 2). These were accessions number 1072, 1179, 1184 and 1187 (Appendix 4).

In 2009, the lengths of 3 leaves from different plants (Chapter 2) within each

selected plot were assessed. Significant differences in the leaf lengths of accessions were found (p value below 0.001). Leaf lengths ranged from 13 to 19 cm. Accessions 1001, 1043, 1256 and 1260 had leaf lengths above 18 cm. Accessions 1140, 1179 and 1245 had leaf lengths below 14 cm.

In 2009, the lengths of 3 inflorescences from replicate different plants (Chapter 2) within each selected accession were assessed. Significant differences in the inflorescence lengths of the accessions were found (p value below 0.001). Inflorescence length was found to vary from 6 to 14 cm. Accessions 1012, 1077 and 1256 had inflorescence lengths above 13 cm. Accessions 1013, 1140 and 1262 had inflorescence lengths below 7 cm.

In 2009, the number of leaves on a stem from different plants (Chapter 2) within each selected plot was assessed. Significant differences in leaf number were found (p value below 0.001). There were 6-14 leaves per stem and 18-26 leaflets per leaf. Accessions 1007, 1043, 1104, 1230, 1245 and 1259 had more than 12 leaves per stem. Accessions 1140 and 1179 had less than 8 leaves per stem.

The number of individual inflorescences per stem was also assessed on 3 different replicate plants. The accession effect was found to be significant with p value inferior of 0.001. There were between 5 and 14 inflorescences per stem. Accessions 1013, 1071, 1140 and 1179 had less than 6 inflorescences per stem. Accessions 1256 and 1245 had more than 10 inflorescences per stem with 1245 having 14 inflorescences per stem.

Leaflet number was also evaluated on 3 different leaves from 3 different plants. Accession factor was found to be significant with p value <0.001. There were between 18 and 28 leaflets per leaf. Accessions 1007, 1103, 1104, 1110, 1220 and 1260 had less than 20 leaflets per leaf. Accessions 1001, 1005, 1013, 1028 and 1262 had more than 27 leaflets per leaf (Appendix 4).

Three leaflets were measured in terms of length and width on 3 different plants (Chapter 2). The accession factor was found to be significant for the ratio leaflet length/width (p value <0.001). The leaflet length/width ratio ranged from 2.5 to 4.25. This ratio was dependant on the leaflet shape. The larger the ratio, the more elongated the leaflet. Accessions 1103, 1104, 1169, 1213 and 1220 were characterised by a ratio below 3 (rounded leaflet). Accessions 1005, 1163 and 1241

were characterised by a ratio greater than 4 (elongated leaflet).

In 2008, stem thickness of all accessions was evaluated with a scoring method. In 2009, the thickness of 3 stems from different plants from selected accessions was measured. Accession factor was significant in both cases (p value <0.001 in 2008 and 2009). Stem thickness ranged from 3 to 9mm. Accessions 1140, 1179 and 1256 had stems less than 5mm thick. Accessions 1018 and 1043 had stems larger than 8mm thick. In 2008, more accessions were measured. Accessions 1005, 1016, 1045, 1124, 1133, 1142, 1177, 1185, 1196, 1218, 1252, 1289, 1291 and 1292 were scored below 3 so had the thinnest stems. Accessions 1026, 1041, 1101, 1132, 1168, 1175, 1189, 1193, 1210, 1233 and 1266 had the thickest stems (scored above 6).

The number of stems on 3 different plants was counted for the selected accessions in 2009 (Chapter 2). Accessions factor was found to be significant with p value <0.001. There were from 16 to 48 stems per plant. Accessions 1043, 1077, 1103, 1110, 1157, 1245, 1259 and 1260 had less than 18 stems per plant. Accessions 1017, 1019, 1140, 1179 and 1262 had more than 30 stems per plant, with 1140 and 1179 having more than 46 stems per plant.

Three stems on 3 different plants were measured for each plot. Accessions had significantly different stem length (p value <0.001). Stem length ranged from 59 to 105 cm. Accessions 1012, 1043, 1266 and 1290 had stems longer than 100 cm. Accessions 1140, 1165 and 1179 had stems shorter than 75 cm.

4.2.2. Traits correlated with colour

In 2008, all accessions were scored between 3 and 7 for the dominant flower colour. In 2009, 3 flowers of each plot (harvested randomly) were scored for their colour between 1 and 3. Both years, accession effect was found to be significant (p value <0.001). In 2008, accessions 1007, 1026, 1153, 1175, 1183, 1213, 1219, 1220 and 1264 were scored for having very light flower colour (score below 3). Accessions 1008, 1046, 1072, 1110, 1139, 1143, 1160, 1179, 1185, 1198, 1248, 1249, 1250, 1253 and 1256 were scored in 2008 for their darker flower (score above 6).

In 2008, all accessions were scored between 3 and 7 for the dominant stem colour. In 2009, only 3 stems of each plot (harvested randomly) were scored for their colour between 1 and 9. Both years, accession effect was found to be significant (p value <0.001).

Stems were mostly green (1 to 5), only a few were red (5 to 8). Accessions with red stems (score above 5) in 2008 were 1106, 1132, 1176, 1185 and 1213, and in 2009 were 1179, 1197 and 1245 (Appendix 4).

In 2008, all accessions were scored between 3 and 7 for the dominant leaf colour. Accession effect was found to be non-significant and therefore was not evaluated again in 2009 (p value = 0.5).

4.3. Correlations between morphological traits of *O. viciifolia* germplasm

A correlation analysis between morphological traits was performed using Genstat. Inflorescence number per stem is significantly correlated with leaf number per stem, inflorescence length and stem number per plant (respectively p value 7.2 x 10^{-8}, 0.03 and 0.001, r (correlation coefficient) = 0.72, 0.34 and -0.48).

Leaf number per stem is significantly correlated with inflorescence length, leaf length, stem length, stem thickness and stem number per plant (respectively p value = 0.01, 0.01, 0.001 and 0.02, r = 0.39, 0.38, 0.48 and 0.36).

Stem length is correlated with stem thickness and stem number per plant (respectively p value = 0.002 and 0.0004, r = 0.47 and -0.52).

Inflorescence length is significantly correlated with leaf length, leaflet number per leaf, leaflet ratio, stem length and stem number per plant (respectively p value = 0.006, 0.002, 0.02, $2x10^{-5}$ and 0.0003, r = 0.42, -0.46, -0.35, 0.61 and -0.53).

Leaf length is significantly correlated with stem length and stem number per plant (respectively p value = 0.006 and 0.03, r = 0.42 and -0.34).

Leaflet number per leaf is significantly correlated with leaflet ratio and stem length (respectively p value = 0.01 and 0.03, r = 0.40 and -0.33).

4.4. Effect of geographic and climatic origins on *O. viciifolia* morphological traits

REML analyses were performed to test if the geographical origin (country, climate or biome) was a strong determinant of the germplasm diversity (Section 3.6.). Koppen 1 zones explained the most of the traits; the environmental conditions seen in other geographical zones also explained the morphological traits to some extent (Table 25).

Table 25: Significant relations between geographic origin and morphological traits

Trait	**Source of variation**	**F pr.**
Inflorescences per stem	biome	0.03
	country	0.047
Leaves per stem	biome	0.002
	koppen1	0.004
	koppen2	0.019
Inflorescence length	koppen1	0.042
Number of leaflets	country	<.001
Stem length	koppen1	0.037
Stems per plant	biome	0.002
	koppen1	0.005
	koppen2	0.003

Accessions from temperate grasslands were characterised by a high number of inflorescences and leaves per stem, but a low number of stems. In opposition, accessions from temperate conifer areas were characterised by a low number of inflorescences and leaves, but a higher number of stems (Table 26). Thus they could be described as “bush” types, a characteristic probably linked to their mountainous origin.

Table 26: Values of traits significantly correlated to the biome climate zone

Biome	**Inflorescences per stem**	**Leaves per stem**	**Stems per plant**
Mediterranean forests, woodlands scrub	8	11	24
Montane grasslands shrublands	8	12	17
Temperate broadleaf mixed forests	8	11	24
Temperate conifer forests	6	7	48
Temperate grasslands, savannas shrublands	10	12	19

Accessions from mountainous climates (polar) were characterised by shorter stems and inflorescences, fewer leaves per stem; but more stems per plant (Table 27).

Table 27: Values of traits significantly correlated to the Koppen 1 climate zone

Koppen1	**Leaves per stem**	**Inflorescence length**	**Stem length**	**Stems per plant**
Arid	11	9.9	89.5	23
Cold	12	11.4	89.2	22
Polar	7	6.8	61.5	48
Temperate	11	9.9	83.2	24

Accessions from Eastern European countries (Czech Republic, Romania, and Russia) were characterised by more inflorescences per stem. In contrast, accessions from Western European (Germany, Norway, Spain, Switzerland, and UK) were characterised by more leaflets per leaf (Table 28).

Table 28: Values of traits significantly correlated to the country of origin

Country	Inflorescences per stem	Number of leaflets
Armenia	8	21
China	8	19
Czech Republic	9	20
Former Soviet Union	8	23
France	8	25
Germany	8	24
Iran	7	21
Italy	7	22
Kazakhstan	14	24
Lithuania	7	24
Morocco	10	20
Norway	7	24
Poland	7	22
Romania	8	22
Russia	8	22
Slovakia	7	23
Spain	7	24
Switzerland	7	23
Turkey	9	20
UK	8	26
USA	7	21

4.5. Differences between wild and cultivated accessions

Significant differences according to the domestication status, as indicated by whether the lines were derived from wild populations or from lines that had in theory undergone plant genetic improvement through selection and breeding, were only found for the number of leaflets and the stem thickness (Table 29).

Table 29: Significant relations between cultivation status and morphological traits

Trait	**F pr.**
Number of leaflets	0.028
Stem thickness	< 0.001

Wild accessions were characterised by a lower number of leaflets and thinner stems (Table 30).

Table 30: Values of traits significantly correlated to the cultivation status

Status	**Number of leaflets**	**Stem thickness**
Cultivar	23	3.5
Cultivated	24	3.2
Wild	21	2.8

4.6. Cluster analysis

A cluster analysis incorporating all morphological traits and the flowering data in 2009 was performed using GenStat. Two coherent clusters were revealed (>50% different), indicating a clear separation between Western European accessions and accessions from the rest of the world (Figure 35).

A REML analysis was then performed by separating these two clusters. Most traits were found to be significantly different. Eastern European accessions were characterised by later flowering, longer stems, leaves and inflorescences, higher number of inflorescences but lower number of stems per plant (Table 31). These contrasting traits could be linked to major seasonal differences and past agricultural uses.

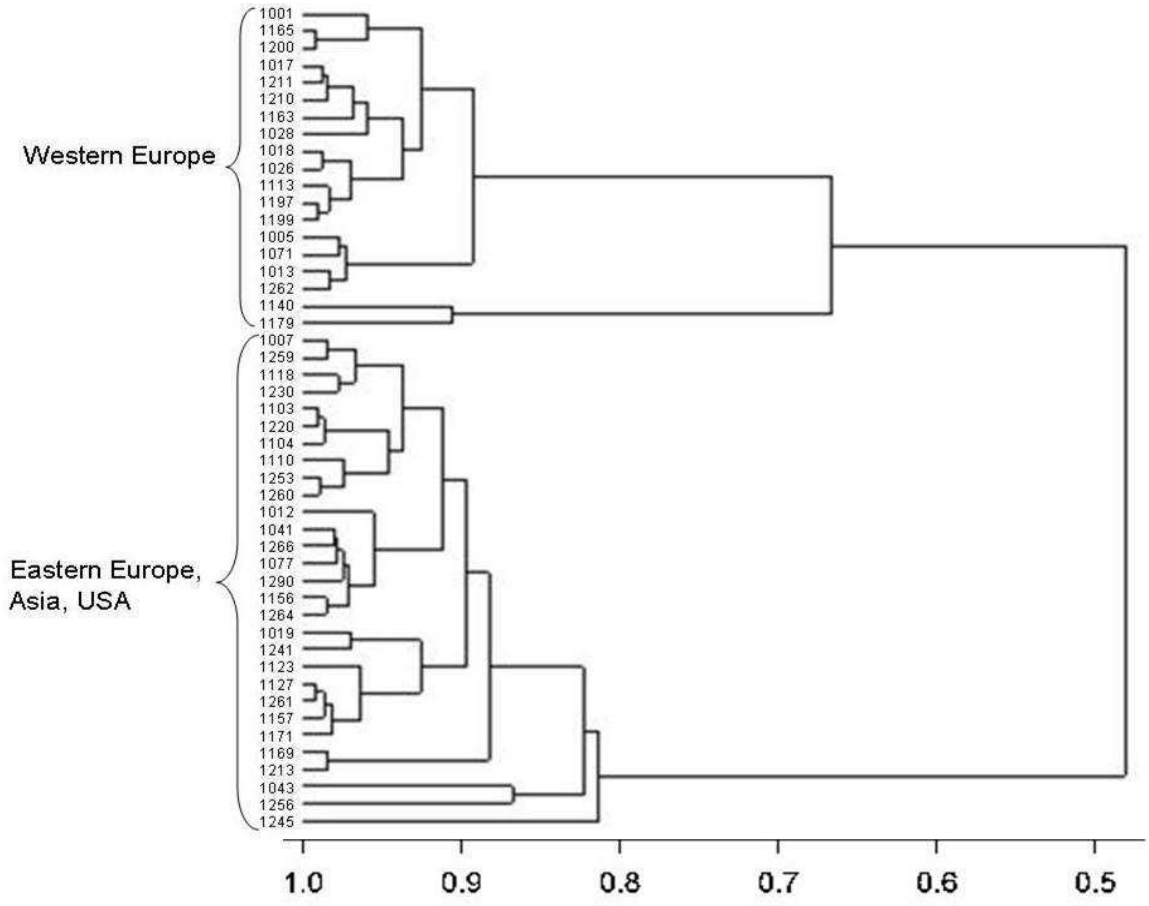

Figure 35: Complete linkage analysis using flowering date (2009) and all morphological traits.

Table 31: REML analysis of the morphological traits between Eastern European countries and the rest of the world.

	Flowering date	Stem length	Inflo. per stem	Leaves per stem	Inflo. length	Leaf length	Number of leaflets	Leaflet length/ width	Stems per plant
Western Europe	140	82	7	10	9	15	25	3.6	29
Rest of the world	146	91	8	11	11	16	21	3.3	21
Significance	<0.001	<0.001	0.02	0.005	<0.001	0.016	<0.001	0.002	<0.001

4.7. Image analysis of *O. viciifolia* seeds

Seeds were characterised by image analyses. Software (developed by Mr R. Farrell, NIAB) was used to measure several seed morphometric traits. Areas were measured in cm^2, length and width in mm. Indexes were created to assess the shape of seeds. The shape index measurement is based on the idea of boundary length to area, normalised so that a circle has a shape of 1.0 and anything else has a shape of < 1.0. The shape change gives a measure of the irregularity of the seeds (e.g. spines), the higher it is the more indented the seed is. All of these values are given in Appendix 5 for the 75 accessions. These morphometric traits were all found to be significantly different among the 75 accessions ($p < 0.001$).

Cultivation status has a significant effect on seed area and dimensions. Seeds from wild accessions were longer and their area is greater than seeds from cultivated/cultivar accessions (Table 32).

Table 32: Means for area, length and width/length ratio according to cultivation status

	Cultivar	**Cultivated**	**Wild**	**F pr**
Area cm^2	0.2384	0.2611	0.273	0.012
Width/length	0.7305	0.7113	0.7055	0.014
Length mm	6.66	7.081	7.237	0.005

The geographic origin of each accession was shown to have a significant effect on the width to length ratio (Table 33). Accessions from China, Hungary and the USA appear to have a reduced length to width ratio, thus to be more elongated. Accessions from Bulgaria, Switzerland and the UK have a high length to width ratio, thus to be more rounded.

Table 33: Means of the width to length ratio according to countries

Country	**F pr=0.034**
Armenia	0.7166
Bulgaria	0.7855
China	0.6758
Czech Republic	0.713
Former Soviet Union	0.7166
Germany	0.7118
Hungary	0.6909
Iran	0.7024
Italy	0.7216
Lithuania	0.7137
Morocco	0.7085
Norway	0.704
Poland	0.7166
Romania	0.7197
Russia	0.7077
Spain	0.7097
Switzerland	0.7399
Turkey	0.7052
UK	0.7309
Ukraine	0.7242
USA	0.6787

All other morphometric characteristics and accession origin data were not found to be statistically significant.

A linkage tree analysis was performed. Four different clusters were given by this analysis (Figure 36). Clusters were not found to be generally linked to the geographical or climatic origin or the cultivation status. The upper cluster is made of mostly of cultivated accessions from the UK.

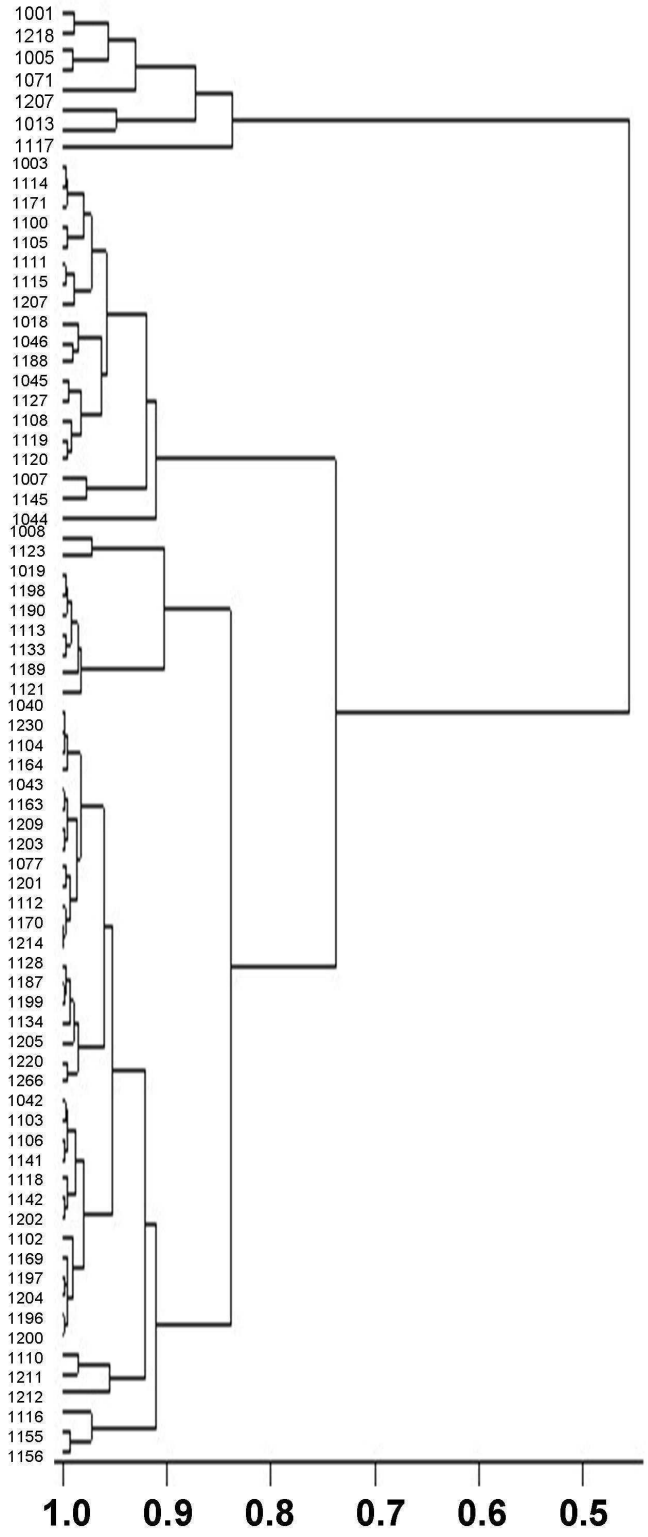

Figure 36: Linkage similarity tree based on all morphometric traits measured on the seeds from the 75 accessions.

These different clusters probably countain seeds possessing similar morphometric characteristics (small versus big - see Figure 37 for an example or - spiked versus rounded) that are not related to the accession origin.

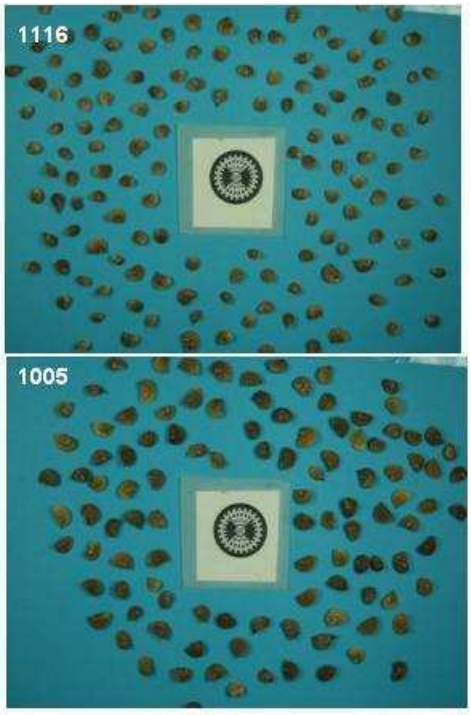

Figure 37: Comparison of small seeds (accession 1116) and large seeds (accession 1005) showing that important variations in seed size can greatly affect the result of image analyses

4.8. Discussion

As observed for the agronomic traits, morphological traits were highly variable among the accession. Morphological characterisation was also a challenging task as reproducibility of measurements was crucial but difficult because of the intra-accession variability. Very significant differences were observed in colour shape and size of the different organs. The most unusual individuals were characterised by white flowers. A few accessions were characterised by red stems or totally prostrate habit, although these traits were sparsely found in some other individuals. A high morphological variability was also observed among a limited germplasm from central Italy (Negri and Cenci, 1988).

Diversity in leaf shape and length can be important from an agronomic perspective, as it can help to decide whether landraces are more adapted to pasture, hay production or silage. Colour diversity might be linked to the polyphenolic composition and/or quantities, and therefore be a good indicator of specific beneficial properties (e.g. anti-bloat, anthelmintic). The normal distributions observed in morphological traits shows that similarly to the agronomical traits, only a few accessions were characterised by extreme values. These traits might be of interest for breeding purposes.

Significant differences in morphological traits were seen between accessions of the germplasm collection. Few correlations with geographic and climatic origin were found probably due to the limited accession number analysed. Still, solid correlations were shown on crucial traits, and it appears that geographic origin was a strong driver of *O. viciifolia* morphological characteristics. A clear division (more than 50% of differences) was found between Eastern European accessions, which were characterised by more inflorescences, and Western European ones characterised by more leaflets. This might reflect general agricultural uses with pasture dominating in Eastern Europe, and hay production, with preference to foliage production, in Western Europe.

The most striking morphological difference was shown for mountainous accessions. These accessions were characterised by lower organ length but higher number of stems. This reduced organ size was also recorded for mountainous accessions in a germplasm from central Italy (Negri and Cenci, 1988). This is a typical adaptation to mountainous condition which has been observed for a quantity of alpine plants. These morphological features are due to the shorter warm season and important UV and temperature stresses. Mountain-adapted *O. viciifolia* constitute a very important forage option as they could be optimal forages for pasture in mountainous zones affected by drought and poor soils.

Cultivated and cultivar accessions were characterised by thick stems and a higher number of leaflets. These characteristics were probably selected for hay production and silage, because such characteristics allow production of more biomass.

The cluster analysis, combining all morphological traits and flowering data, strongly support (more than 50% difference between the two clusters) a general

distinction between Western European accessions and accessions from the rest of the world. This distinction might be explained by climatic factors as the accessions from Western Europe, generally characterised by a temperate climate, tends to have an earlier flowering date. There might also be an effect of agricultural history. Accessions from Western Europe were characterised by a greater number of thick stems with more leaflets, characteristics that have probably been selected for hay production or silage. Other accessions were characterised by longer organs and higher numbers of leafs and inflorescences, characteristics probably more adapted for livestock pasture.

As observed during the germplasm collection, seeds were very variable in size and shape. Most morphometric traits were found to differ significantly among these accessions. Seeds derived from certain countries (China, Hungary, USA) were more elongated compared to seeds from other countries (Bulgaria, UK and Switzerland) which were more rounded, but no coherent cluster was found. A significant trend was shown for wild accessions to have bigger seeds. This might be crucial for wild accessions as establishement is made difficult by competition from other plants. Thus larger nutrient stocks are necessary to ensure some germination success. Different groups of seeds were shown to exist, based mainly on varying size and shape. Somewhat surprisingly, this variation does not seem to be linked to the origin and cultivation status of different *O. viciifolia* accessions.

CHAPTER 5. CYTOLOGICAL CHARACTERISATION OF *ONOBRYCHIS* GENUS

The ploidy level of *O. viciifolia* was checked as well as the chromosome number of other *Onobrychis* species. The genome size of *O. viciifolia* was also investigated.

5.1. Ploidy level of *Onobrychis viciifolia*

Some work was necessary to clarify the ploidy level of *O. viciifolia* (Chapter 1).

5.1.1. Flow cytometry screening of *Onobrychis viciifolia* accessions

Flow cytometry is a fast and accurate method for evaluating ploidy level (Chapter 1).

The flow cytometry methodology is described in Chapter 2. Graphs were obtained for all accessions. The ploidy level was determined by comparison with the reference tetraploid *O. viciifolia* accession 1127. An example of the shift in profiles observed between diploid and tetraploid accessions is shown in Figure 38. Peaks corresponding to higher (G2 mitotic phase) and lower (G1 mitotic phase) DNA cell content are detected separately, with a direct correlation between DNA content and relative fluorescence. This correlation results in shifted peaks when ploidy level (and thus DNA content) is doubled.

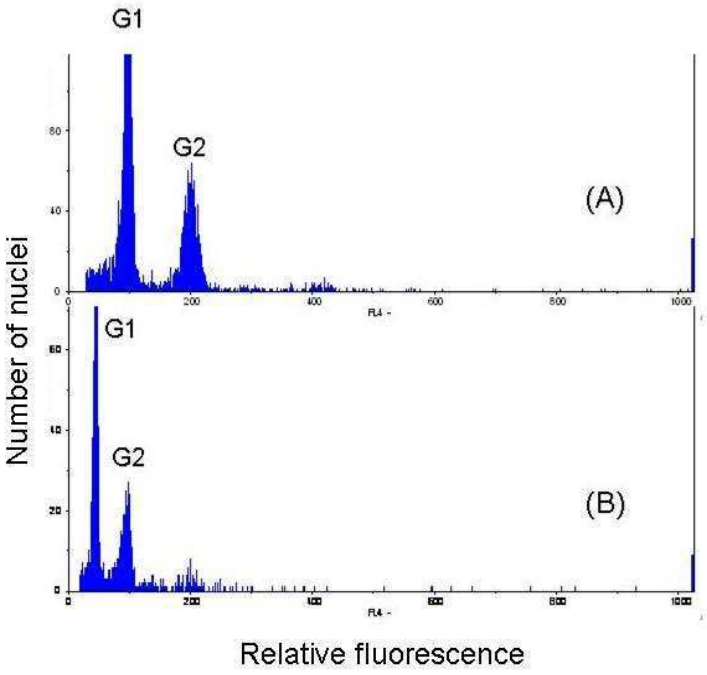

Figure 38: Profiles obtained by flow cytometry, using 4', 6-diamidino-2-phenylindole fluorophore, showing shifted G1 and G2 mitotic phase peaks of (A) tetraploid 1001 accession and (B) diploid 1126 accession

The ploidy level results obtained are summarised in Table 34 below.

Table 34: Ploidy level of *Onobrychis viciifolia* accessions as determined by flow cytometry.

Accession	**Ploidy level**	**Accession**	**Ploidy level**
1001	Tetraploid	**1128**	Tetraploid
1002	Tetraploid	**1130**	Tetraploid
1003	Tetraploid	**1131**	Tetraploid
1004	Tetraploid	**1137**	Tetraploid
1005	Tetraploid	**1148**	Tetraploid
1006	Tetraploid	**1150**	Tetraploid
1007	Tetraploid	**1152**	Tetraploid
1008	Tetraploid	**1153**	Tetraploid
1009	Tetraploid	**1154**	Tetraploid
1010	Tetraploid	**1155**	Tetraploid
1011	Tetraploid	**1156**	Tetraploid

1012	Tetraploid	**1157**	Tetraploid
1013	Tetraploid	**1158**	Tetraploid
1014	Tetraploid	**1160**	Tetraploid
1017	Tetraploid	**1161**	Tetraploid
1018	Tetraploid	**1162**	Tetraploid
1019	Tetraploid	**1163**	Tetraploid
1021	Tetraploid	**1164**	Tetraploid
1026	Tetraploid	**1165**	Tetraploid
1027	Tetraploid	**1166**	Tetraploid
1028	Tetraploid	**1167**	Tetraploid
1029	Tetraploid	**1168**	Tetraploid
1030	Tetraploid	**1169**	Tetraploid
1031	Tetraploid	**1170**	Tetraploid
1032	Tetraploid	**1179**	Tetraploid
1033	Tetraploid	**1181**	Tetraploid
1037	Tetraploid	**1197**	Tetraploid
1039	Tetraploid	**1198**	Tetraploid
1040	Tetraploid	**1199**	Tetraploid
1041	Tetraploid	**1200**	Tetraploid
1042	Tetraploid	**1202**	Tetraploid
1043	Tetraploid	**1203**	Tetraploid
1046	Tetraploid	**1205**	Tetraploid
1071	Tetraploid	**1206**	Tetraploid
1073	Tetraploid	**1208**	**Diploid**
1074	Tetraploid	**1210**	Tetraploid
1075	Tetraploid	**1211**	Tetraploid
1076	Tetraploid	**1212**	Tetraploid
1077	Tetraploid	**1213**	Tetraploid
1078	Tetraploid	**1214**	Tetraploid
1079	Tetraploid	**1220**	Tetraploid
1081	Tetraploid	**1223**	Tetraploid
1082	Tetraploid	**1224**	Tetraploid

1083	Tetraploid	**1225**	Tetraploid
1084	Tetraploid	**1226**	Tetraploid
1085	Tetraploid	**1227**	Tetraploid
1086	Tetraploid	**1228**	Tetraploid
1087	Tetraploid	**1229**	Tetraploid
1088	Tetraploid	**1230**	Tetraploid
1089	Tetraploid	**1231**	Tetraploid
1091	Tetraploid	**1232**	Tetraploid
1092	Tetraploid	**1233**	Tetraploid
1093	Tetraploid	**1234**	Tetraploid
1094	Tetraploid	**1235**	Tetraploid
1096	Tetraploid	**1240**	Tetraploid
1097	Tetraploid	**1241**	Tetraploid
1098	Tetraploid	**1244**	Tetraploid
1099	Tetraploid	**1246**	Tetraploid
1103	Tetraploid	**1248**	Tetraploid
1104	Tetraploid	**1252**	Tetraploid
1105	Tetraploid	**1253**	Tetraploid
1106	Tetraploid	**1256**	Tetraploid
1109	Tetraploid	**1257**	**Diploid**
1110	Tetraploid	**1258**	Tetraploid
1113	Tetraploid	**1260**	Tetraploid
1116	Tetraploid	**1261**	Tetraploid
1117	Tetraploid	**1262**	Tetraploid
1118	Tetraploid	**1266**	Tetraploid
1123	Tetraploid	**1290**	Tetraploid
1125	Tetraploid	**1291**	Tetraploid
1126	**Diploid**	**1292**	Tetraploid
1127	Tetraploid		

Most of the *O. viciifolia* accessions selected for this study were tetraploid, and all the accessions that are cultivated in the NIAB field were found to be

tetraploid. There were only three diploid accessions in the set. Thus most of the *O. viciifolia* are tetraploid but a very limited diploid proportion exists and their geographic origin is diverse (Greece, former Soviet Union and Turkey).

5.1.2. Microscopic observations of chromosome numbers for *Onobrychis* species

Microscopic observation of meristematic root tissue was performed on selected *O. viciifolia* and other *Onobrychis* species to clarify and confirm the chromosome counts obtained by flow cytometry. The method is described in Chapter 2.

5.1.2.1 Microscopic observation of *Onobrychis viciifolia* meristematic root tissue

The flow cytometry results obtained with *O. viciifolia* were further checked by microscopic observation of meristematic root tips. This enabled confirmation that the chromosome numbers in tetraploid accessions was 2n=4x=28 and in diploid accessions was 2n=2x=14.

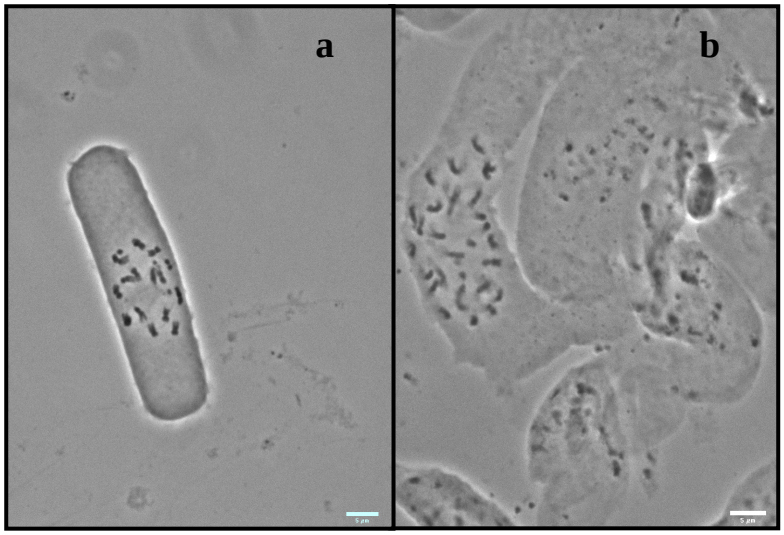

Figure 39: Stained metaphasic *Onobrychis viciifolia* meristematic root cell chromosomes, a) diploid cell with 2n=2x=14 chromosomes from accession 1257 and b) tetraploid cell with 2n=4x=28 from accession 1292

Diploid root cells with 14 chromosomes and tetraploid root cells with 28

chromosomes were observed (Figure 39).

Ploidy levels observed by microscopy always confirmed the flow cytometry results. Chromosomes were very small (<5μm).

5.1.2.2. Chromosome numbers of *Onobrychis* species observed by meristematic root cell microscopy

Information on the chromosome number and ploidy of *Onobrychis* species is scarce and often contradictory in the literature (Chapter 1). The chromosome number of a variety of species was therefore evaluated by microscopy (ploidy level could not be determined accurately by flow cytometry due to the lack of a reference accession).

Results of these observations are listed in Table 35, and compared to the chromosome numbers found in the literature. The results generally agreed with the published information. In some cases differences might be due to differences between the accessions that were characterised. One major difference was found in the case of *O. bungei*, for which twenty eight chromosomes were observed and only sixteen were reported by the reference.

Table 35: Ploidy level of 16 *Onobychis* species evaluated by microscopy. Where available, the chromosome number given in the literature is indicated in brackets in column 3

Species	**Section**	**Chromosomes**	**Reference**
O. altissima	Onobrychis	28	NA
O. antasiatica	Onobrychis	28	NA
O. arenaria	Onobrychis	14/28 (14/28)	http://www2.dijon.inra.fr/flore-france/oa-oo.htm
O. biebersteinii	Onobrychis	28 (28)	(Kidambi *et al.*, 1990a)
O. bungei	Onobrychis	28 (16)	http://www.agroatlas.ru/en/content/related/Onobrychis_bungei/
O. cyri	Onobrychis	28 (28)	http://www.agroatlas.ru/en/content/related/Onobrychis_cyri/
O. gracilis	Onobrychis	28 (28)	(Pavlova and Manova, 2000)
O. iberica	Onobrychis	28 (28)	http://www.agroatlas.ru/en/content/related/Onobrychis_iberica/
O. montana	Onobrychis	28 (14/28)	(Pavlova and Manova, 2000; Tamas, 2006)
O. petraea	Onobrychis	14 (14)	(Kidambi *et al.*, 1990a)
O. transcaucasica	Onobrychis	28(28)	(Kidambi *et al.*, 1990a)
O. aequidentata	Lophobrychis	16 (14/16/28)	(Abou-El-Enain, 2002)
O. alba	Lophobrychis	14/28 (14/32)	(Abou-El-Enain, 2002)
O. crista-galli	Lophobrychis	16 (14/16/32)	(Abou-El-Enain, 2002)
O. radiata	Hymenobrychis	14 (14)	http://cat.inist.fr/?aModele=afficheN&cpsidt=17846696
O. subacaulis	Heliobrychis	14	NA

5.2. Genome size evaluation

Genome size was determined using flow cytometry as described in Chapter 2. Propidium iodide (PI) and 4', 6-diamidino-2-phenylindole (DAPI) were both used as fluorophores. *Zea mays* was found to be the best standard available for DNA content determination. *Zea mays* L. CE-777 (2C=5.43pg) was used as a reference and run simultaneously with *O. viciifolia* samples. Genome size was calculated according to the formula:

$$\text{Sample 2C value} = \frac{\text{Reference 2C value x Sample G1 mean peak position}}{\text{Reference G1 mean peak position}}.$$

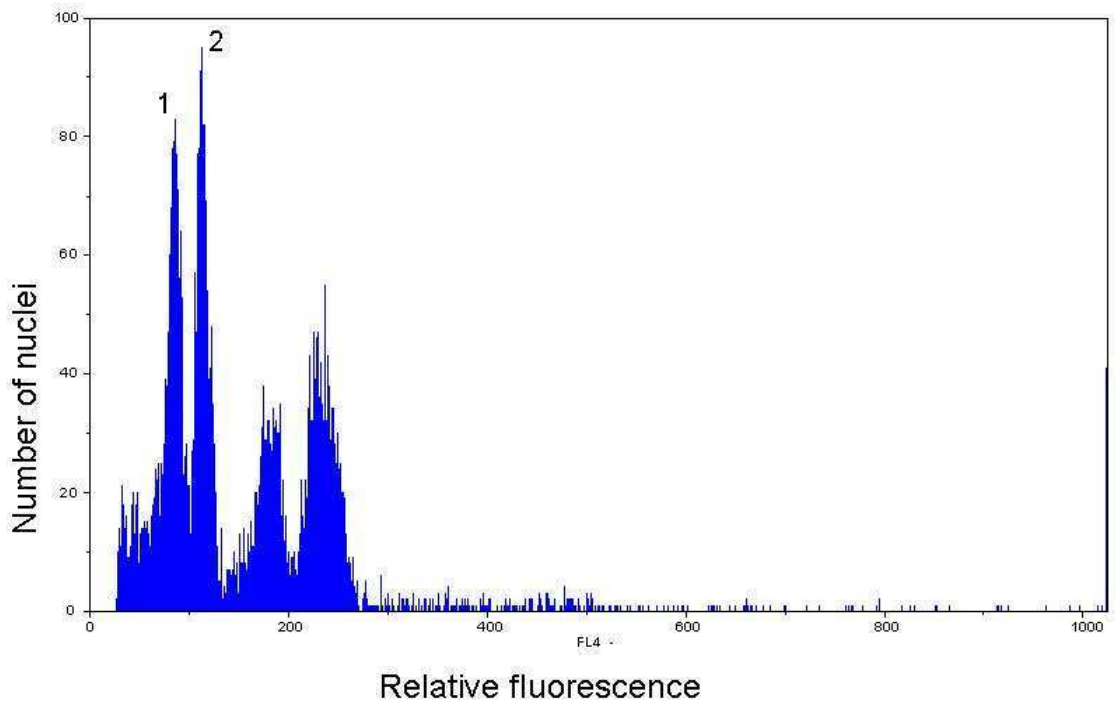

Figure 40: Profiles obtained by flow cytometry using 4', 6-diamidino-2-phenylindole fluorophore combining *Zea mays* L. 'CE-777' and *O. viciifolia* samples (1 and 2 correspond to G1 mitotic phase peak *O. viciifolia* 1001 accession and *Zea mays* respectively). The other peaks correspond to the G2 phases

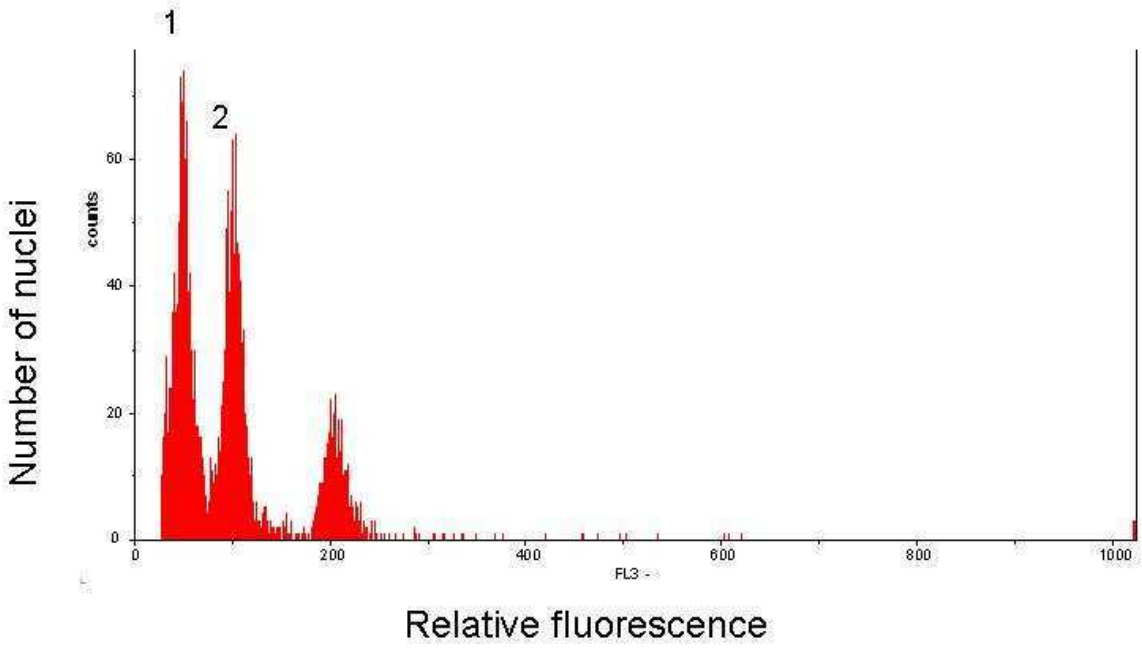

Figure 41: Flow profiles obtained by flow cytometry using propidium iodide fluorophore combining *Zea mays* L. 'CE-777' and *O. viciifolia* samples (1 and 2 correspond to G1 mitotic phase peak *O. viciifolia* 1001 accession and *Zea mays* respectively). The other peak corresponds to the G2 phases

Profiles obtained using DAPI (Figure 40) were clearer than the ones obtained using PI (Figure 41). The two G2 phases could clearly be distinguished. This is probably due to the fact that PI is more sensitive to chromatin conformation (Jaroslav Dolezel, Associate Professor, Department of Cell Biology and Genetics, Palacky University, Olomouc, Czech Republic, personal communication).

The 2C value for *Onobrychis viciifolia* was calculated to be 4 pg with DAPI and 2.5 pg with PI. This difference was expected as DAPI is guanine and cytosine (GC) content sensitive.

5.3. Discussion

The chromosomes as observed by microscopy were found to be very small (<5µm). This is in agreement with observations by Tamas (2006). The chromosomes of other *Onobrychis* species were generally even smaller. This small size makes its difficult to obtain an accurate count of the chromosome number by microscopy, even

at 1000X magnification. In the case of this genus, the value of the cytometry approach was thus very tangible.

From both cytological and microscopic observations, it is clear that the large majority of *O. viciifolia* accessions are tetraploid. Only a few wild accessions were found to be diploid. This is coherent with much of the literature, in which many authors agree that *O. viciifolia* is tetraploid (Kidambi *et al.*, 1990a; Tamas, 2006) or sometimes diploid (http://www.agroatlas.ru/en/content/related/Onobrychis_viciifolia/). Therefore, it appears likely that diploid forms were counter selected naturally or by human use in favour of tetraploids.

Unfortunately, none of the diploid accessions were transferred to plots in the field due to low germination rate (Chapter 2). This confirms the common rule that plants of lower ploidy level are generally less vigorous, and that generally polyploid plants are more successful and more competitive in arable situations (Hegarty and Hiscock, 2008).

The chromosome numbers determined for other *Onobrychis* species generally agreed with observations by other authors (Kidambi *et al.*, 1990a; Pavlova and Manova, 2000; Abou-El-Enain, 2002). A general distinction was seen between the Onobrychis section and the other sections. Where most species from the Onobrychis section were shown to possess 28 chromosomes, most species from other sections were shown to possess 14 or 16 chromosomes. Only *O. petraea* and some *O. arenaria* had 14 chromosomes in the Onobrychis section. Within the Lophobrychis, Hymenobrychis and Heliobrychis sections, only some *O. alba* accessions had 28 chromosomes. The basic number of chromosomes among the *Onobrychis* genus has been shown to be 7 or 8 (Abou-El-Enain, 2002). It is unclear if any of this basic chromosome number set is ancestral. Thus it would appear that the *Onobrychis* genus countains either diploid or tetraploid species, with a clear tendancy to have tetraploidy in the Onobrychis section and diploidy in the other sections. The Onobrychis section contains all of the intensively cultivated species in the genus, mainly *O. viciifolia, O. transcaucasica, O. antasiatica* and *O. arenaria* (http://www.ars-grin.gov/, http://www.agroatlas.ru/en/content/related/, National Academy of Sciences of Armenia).

Generally species from other sections are of far less agricultural importance. It can therefore be suggested that polyploidy has probably occurred due to human

selection for agricultural use among *Onobrychis* genus.

Genome size was determined both with DAPI and PI as fluorophores. DAPI fluorophore binds to adenine and thymine and monocotyledon species as *Zea mays* have higher GC content than dicotyledon species like *O. viciifolia*, but was the only available standard that matched *O. viciifolia* genome size range (Karlin and Mrazek, 1997). Therefore, the genome size of *O. viciifolia* was over estimated using DAPI chlorophore and only the value of 2.5 pg (2C) should be considered as representative. This genome size has been submitted to the Kew plant DNA C-values database (http://data.kew.org/cvalues/) and will be available for further studies on *O. viciifolia*.

CHAPTER 6. FINGERPRINTING OF *ONOBRYCHIS VICIIFOLIA* ACCESSIONS

AFLP and SSR methods were developed for *O. viciifolia*.

6.1. DNA extraction

DNA was extracted using three different methods and the quality was checked by Nanodrop spectrophotometry, gel quantification and PCR amplification.

6.1.1. Accessions extracted

In some cases, extractions were performed on different individuals from the same accession. The three different methods of DNA extraction each allowed DNA to be obtained from *O. viciifolia*, other *Onobrychis* species and *Lotus corniculatus* with no apparent difficulties resulting from the high polyphenolic content. The quality of the DNA obtained by the different methods was assessed, mainly in order to determine if the Tanksley method (which is less expensive) could be routinely used for genetic studies of these plants.

The DNA profile of the accessions extracted was cheched on a 1% agarose (w/v) gel (Figure 42).

Figure 42: 1% agarose (w/v) gel representing DNA profiles of accessions 1008, 1112, 1163, 1164, 1179, 1256, 1262 and 1264 (from left to right) extracted with Tanksley protocol with Hyperladder IV from Bioline

6.1.2. DNA quality assessed by spectrophotometry

DNA quality of some representative accessions was checked by spectrophotometry (Nanodrop). This method gives an evaluation of the DNA concentration and of its purity. Two absorbance ratios indicate the DNA purity, the 260nm/280nm ratio indicates the amount of protein and is expected to be greater than 1.8, the best values being 1.8-2.0. DNA with a 260nm/280nm ratio lower than 1.8 is considered contaminated with proteins. The 260nm/230nm ratio indicates the presence of contaminants which absorb at 230 nm (such as carbohydrates and phenol) and is also expected to be more than 1.8, the best values being 1.8-2.2. These measurements for accessions 1008, 1112, 1163, 1164, 1179, 1256, 1262 and 1264, which were chosen for assessement of DNA quality, are listed in Table 36 below.

Table 36: Nanodrop values obtained for the selected accessions (1008, 1112, 1163, 1164, 1179, 1256, 1262 and 1264) with 3 different DNA extraction techniques (Phytopure kit (Phy), Qiagen kit (Qia) and Tanskey modified crude method (Tan))

Sample	**ng/µl**			**A260nm /280nm**			**A260nm/230nm**		
	Phy	**Qia**	**Tan**	**Phy**	**Qia**	**Tan**	**Phy**	**Qia**	**Tan**
1008	1090	75	183	2.2	1.9	1.8	2.1	1.5	0.8
1112	329	44	712	2.2	1.9	1.8	1.9	1.2	0.8
1163	224	25	28	2.2	1.5	1.7	1.7	0.4	0.6
1164	2677	51	43	2.2	1.8	1.7	2.2	1.2	0.6
1179	132	12	21	2.1	1.8	1.8	1.1	0.6	0.5
1256	235	18	25	2.1	2	1.6	1.7	0.7	0.6
1262	953	30	469	2.2	1.9	1.8	2	1	0.9
1264	504	38	63	2.2	1.8	1.8	2.1	1.1	0.6

Better DNA yields were obtained with the Phytopure kit (132-1090ng/µl) and the Tanksley method (21-712ng/µl) compared to the Qiagen kit (12-75ng/µl). All methods gave satisfactory 260nm/280nm ratios with the Tanksley method showing the best values (1.6-2.0), followed by the Qiagen kit (1.5-2.0) and the Phytopure kit (2.1-2.2). The 260nm/230nm ratio was satisfactory only for DNA obtained using the Phytopure kit (1.1-2.2), it was too low with material prepared using the Qiagen kit

(0.4-1.5) and the Tanksley method (0.6-0.9).

6.1.3. DNA quality assessed by PCR amplification

DNA quality was also assessed by PCR amplification using general primers targeting non-coding chloroplastic regions (primer pairs cd and ef, see Chapter 2). Amplification was carried out following recommended protocols (Taberlet *et al.*, 1991) and fragments of the expected size were obtained with DNA obtained using each of the three different methods. These positive amplifications indicated that no significant PCR inhibitors were left by either of the DNA extraction methods.

These observations combined with the Nanodrop results led to the choice of the Tanksley method for extraction of DNA from 40 selected accessions for further fingerprinting and sequence analyses as it was less expensive. These accessions were chosen for their diversity and were also characterised by the morphological analysis (Chapter 2).

6.2. AFLP fingerprinting of *Onobrychis viciifolia*

Fingerpinting of the genotypic diversity of the *Onobrychis* accessions was attempted by AFLP analysis.

6.2.1. Pre-amplification results

Pre-amplification (Chapter 2) was successful. Gel pictures of restriction ligation and pre-amplification are shown in Figure 43 below.

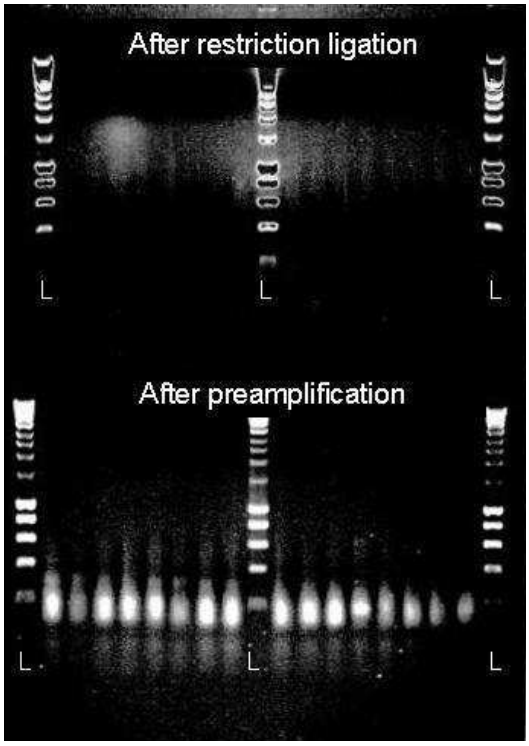

Figure 43: 1% agarose (w/v) gel after the restriction ligation and preamplification stages of AFLP analysis performed with 16 different *Onobrychis viciifolia* accessions. L stands for Bioline Hyperladder I

6.2.2. AFLP method improvement

A first trial was performed by running eight samples on an ABI capillary electrophoresis system. Polymorphic regions were observed, but there were very few peaks and they were very small (160 fluorescence units where at least 1000 would be expected) (Figure 44).

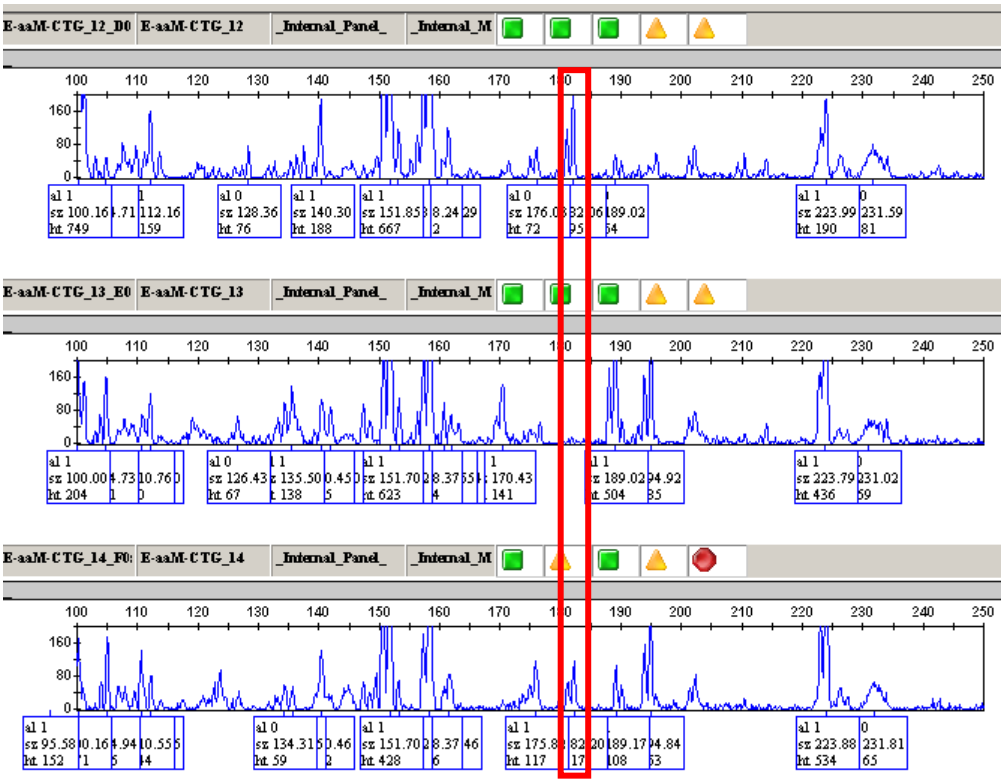

Figure 44: AFLP profiles of three different samples (accessions 1001, 1179 and 1256) with *EcoR1*-AA and Mse1-CTG as selective primers for amplification. Example of polymorphic regions are circled in red

The protocol was improved by reducing the DNA concentration before digestion and then diluting the sample throughout the protocol thus giving a better peak intensity (Figure 45).

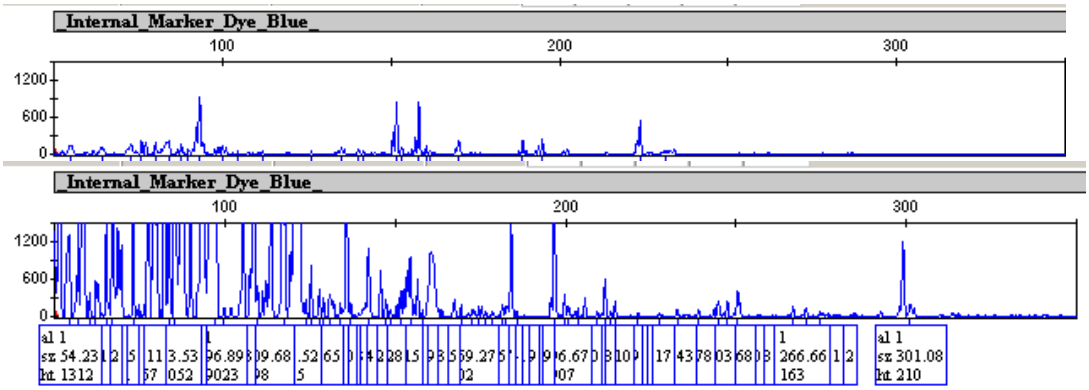

Figure 45: Comparison of the profiles for accession 10001 obtained before (top) and after (bottom) improvements in the AFLP protocol by diluting the DNA allowing a fuller digestion, and reducing the salt contamination

Again, polymorphic regions were observed using the improved protocol (Figure 46).

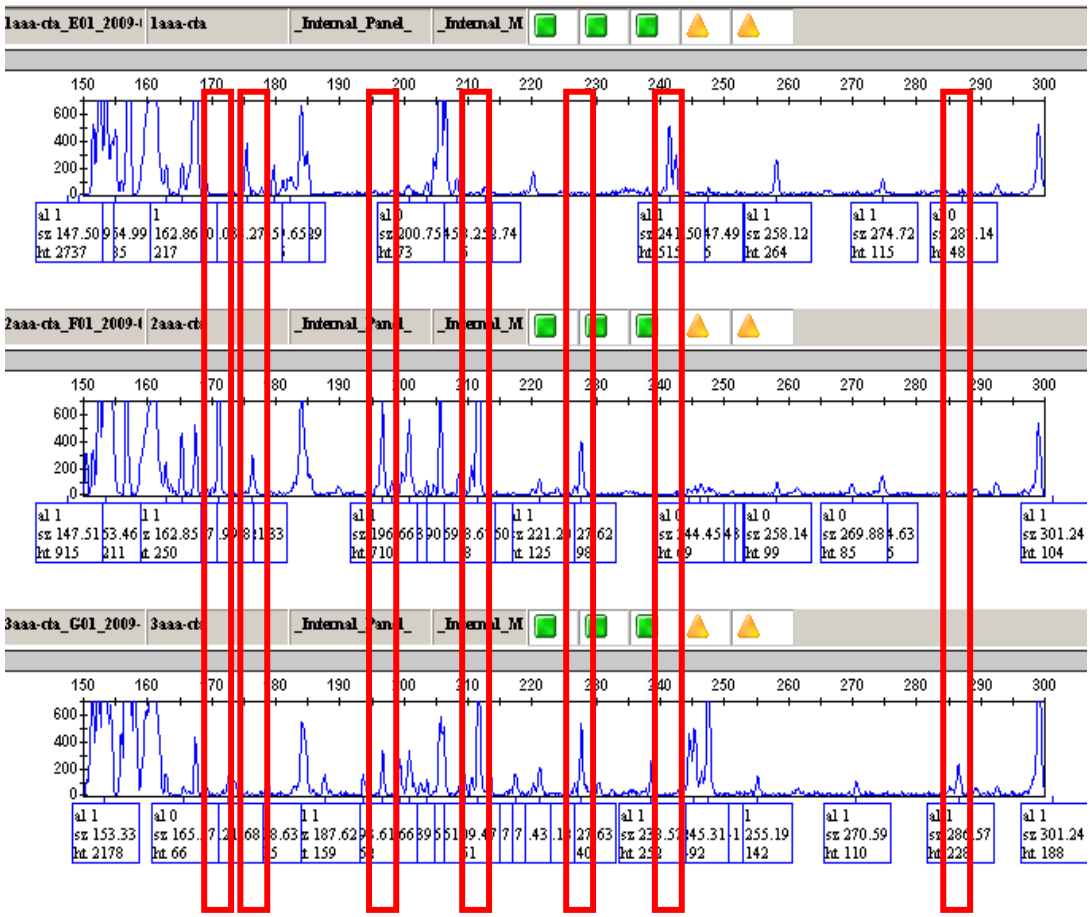

Figure 46: Example of 7 polymorphic regions between different accessions (1001, 1179 and 1256) with one similar selective primer combination *EcoR1*-AAA and Mse1-CTA

Generally, it was clear that different accessions had differences in their profiles, but repeatability was often unsatisfactory. This is illustrated by Figure 47, where accessions 1200 and 1264 possess different general profiles, but there was considerable variability between replicated profiles (DNA extracted from the same plant).

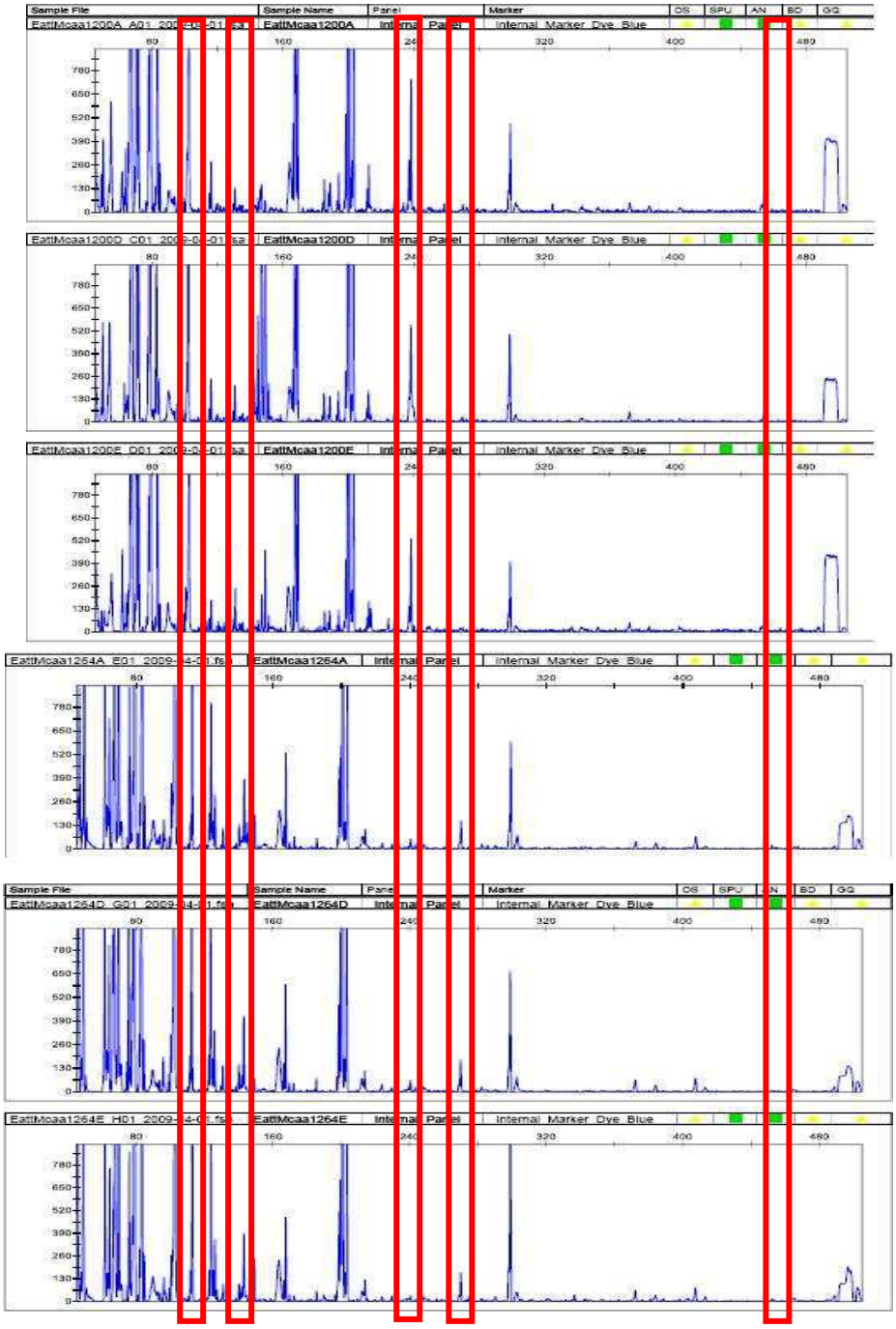

Figure 47: AFLP profiles for repeated samples (DNA extracted from the same plant) of accession 1200 (top 3) and 1264 (bottom 3) using the same selective primer combination *EcoR1*-ATT and Mse1-CAA. Red boxes are examples of polymorphic regions for these two accessions. Size range shown is 0-500 bp

6.3. SSR fingerprinting of *Onobrychis viciifolia*

Fingerpinting of the genotypic diversity of *O. viciifolia* accessions was attempted using SSR analysis.

6.3.1. SSR developpement for *O. viciifolia*

PCRs designed for SSRs from other legumes were used with selected *O. viciifolia* accessions. The best results were obtained with SSR2 (*Medicago* CTT repeat motif), SSR5 (*Medicago* TGAG repeat motif), SSR7 (*Glycine* several repeat motif) and SSR11 (*Medicago* TCC repeat motif). The optimal melting temperatures, the fragment sizes that were obtained and the profile qualities are described in Table 37.

Table 37: SSR markers with optimal melting temperature (Tm), approximate fragment size and profile quality after ABI sequencing (selected markers are represented in bold character)

Marker name	**Optimal Tm**	**Approximate size obtained (bp)**	**Profile quality on ABI**
1	51	Multiple fragments	Not clear
2	**53**	**Multiple fragments**	**Clear**
3	51	600	Not usable with Liz 500
4	55	200	Not clear
5	**53**	**200**	**Clear**
6	53	600	Not usable with Liz 500
7	**55**	**200**	**Clear**
8	53	800	Not usable with Liz 500
9	51	Multiple fragments	Not clear
10	51	400	Not clear
11	**51**	**Multiple fragments**	**Clear**

These SSRs were then applied to the 40 accessions that were chosen for their diversity and also characterised by the morphological analysis (Chapter 2).

Inter-accession variability is illustrated by Figures 48 (SSR2), 49 (SSR5), 50 (SSR7) and 51 (SSR11).

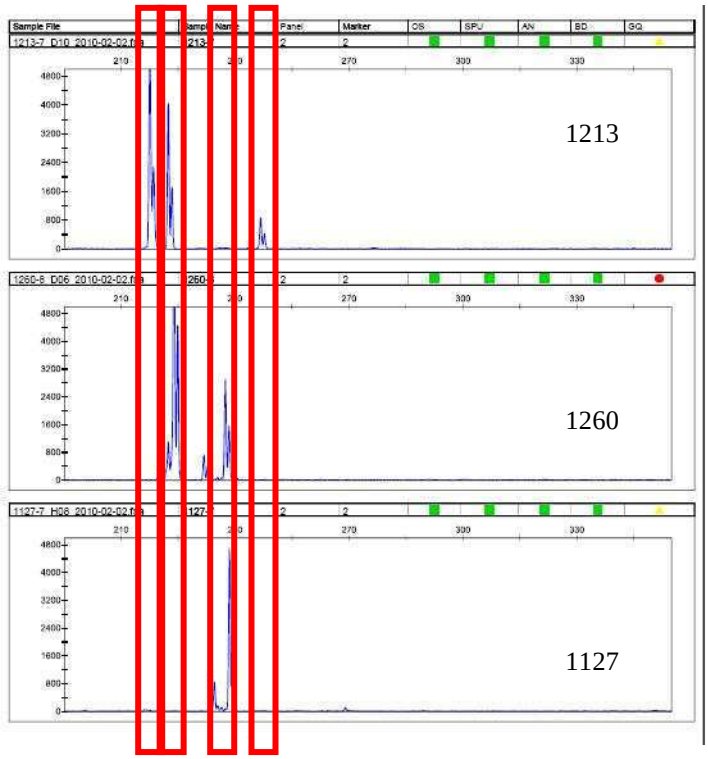

Figure 48: SSR2 accession variability (accessions 1213, 1260 and 1127 are shown as examples with polymorphisms highlighted in red boxes)

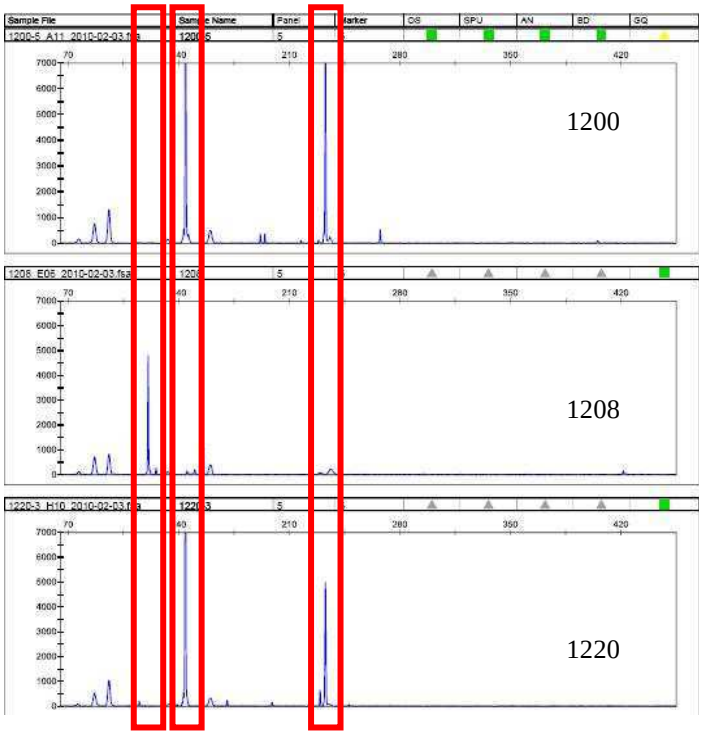

Figure 49: SSR5 accession variability (accessions 1200, 1208 and 1220 are shown as examples with polymorphisms highlighted in red boxes)

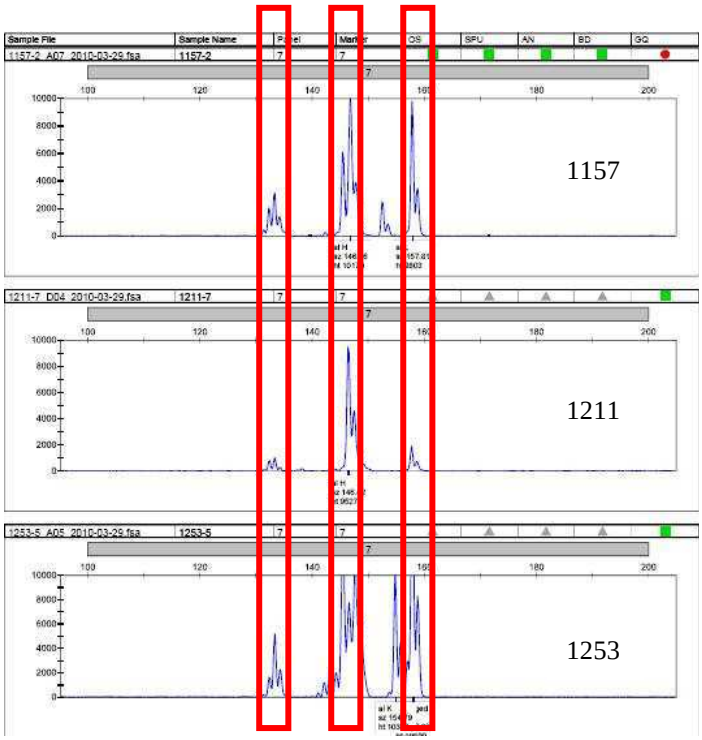

Figure 50: SSR7 accession variability (accessions 1157, 1211 and 1253 are shown as examples with polymorphisms highlighted in red boxes)

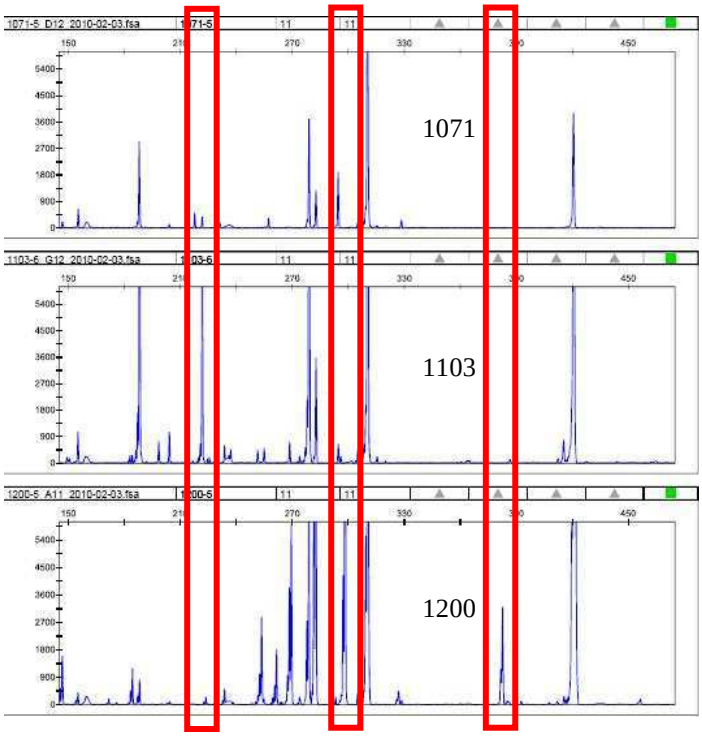

Figure 51: SSR11 accession variability (accessions 1071, 1103 and 1200 are shown as examples with polymorphisms highlighted in red boxes)

6.3.2. High accession variability in SSR profiles

Despite unequivocal differences in the SSR profiles for different accessions and satisfactory repeatability, dissimilarity matrixes based on the four SSR was not sufficient to significantly segregate clusters in the germplasm. The main reason was the high variability between different individuals of the same accession (illustrated below in Figure 52).

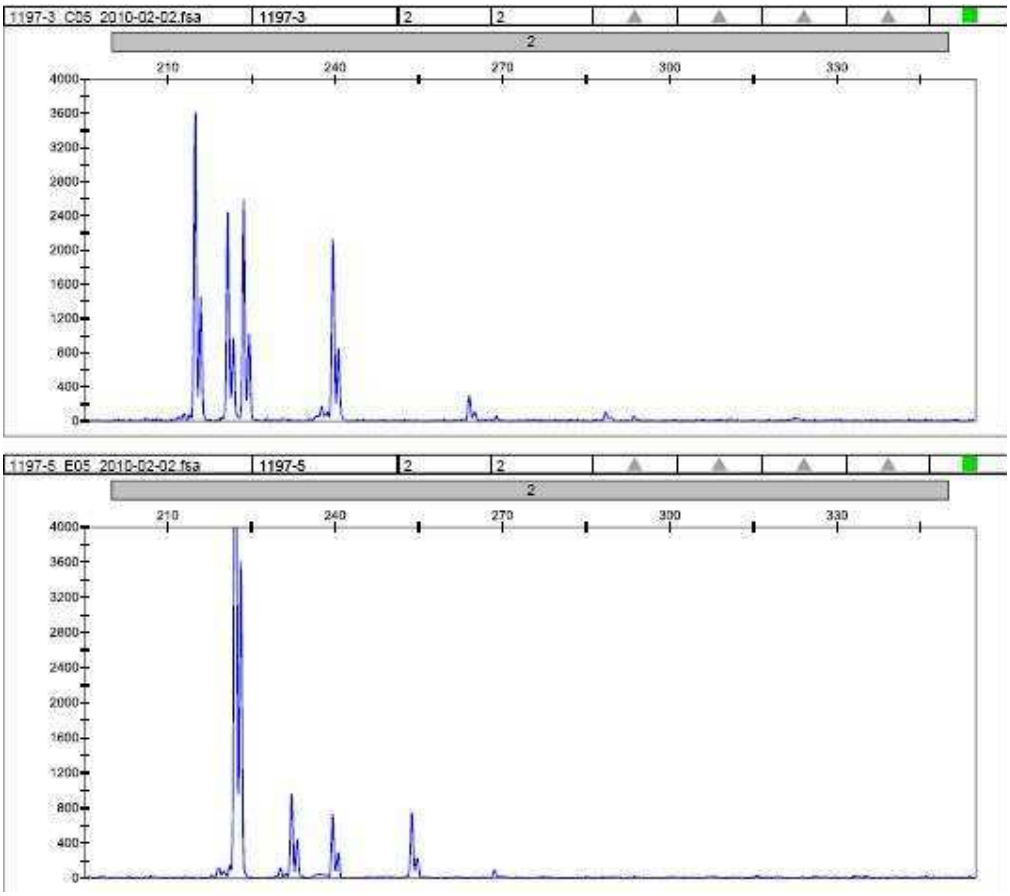

Figure 52: Example of individual variability within an accession. Here the SSR2 profile is shown for 2 individuals of the 1197 accession. Two very different profiles are obtained

More SSRs would have helped to segregate clusters, but time did not allow the search for other SSRs. To improve SSR specificity and avoid unspecific amplification, product sequencing could have been produced in order to find more suitable primers.

6.4. Discussion

Genetic studies on the *Onobrychis* genus have been scarce (Ahangarian, 2007), thus limited knowledge was available on the best methodologies to use. Due to their high tannin content, it appeared very likely that difficulties in DNA extraction might be encountered for *Onobrychis* species.

Accuracy of fingerprinting or sequencing methods is strongly dependent of the quality of DNA obtained. Presence of organic chemicals such as proteins sugars or polyphenols in DNA extracts can greatly inhibit or induce biases to enzymatic reactions. Commercial extraction kits are commonly thought to produce better quality DNA extracts and therefore to be cost-effective. However, crude extraction methods are not necessarily more complicated to perform and are arguably less expensive. Here, the effectiveness of two kits has been compared with the Tanksley modified extraction method. One of the kits was shown to produce lower DNA yield and removal of some organic compounds was unsatisfactorily. The Tanksley method gave DNA of satisfactory quality for PCR based methods eventhough the 230/260 absorbance ratio was low. Furthermore, successful PCR amplifications with general primers showed that no significant inhibitors were present in the DNA extracts. Based on this comparison, the Tanksley method was chosen for further genetic investigations.

AFLP is a commonly used method in plant biology to assess genotypic diversity on a very fine scale, and sometimes allow the development of molecular markers for plant breeding. AFLP protocols generally used for other angiosperms were expected to relatively quickly lead to similar results with *Onobrychis viciifolia*. The AFLP method was apparently effective as polymorphisms were seen after methodological improvements. However, the capillary electrophoresis based approach was probably too sensitive to accurately reflect the results and was probably affected too much by amplification biases or stochastic events. It resulted in non-repeatable profiles even when processing the same products twice. A gel based analysis would have been desirable to decrease the sensivity of the detection method. This was attempted at NIAB using a Li-Cor, but unfortunately, the apparatus did not work correctly. It was not possible to consider alternative approaches within the time constraints of the project. A different detection approach would probably be the best

way to determine genotypic diversity by the AFLP method. Even if not all genetic diversity can be resolved through analysis of major bands, it is highly probable that major diversity patterns would still be easily uncovered. Different digestion time could have been also tested in order to improve the band pattern. For example, Savo-Sardaro *et al.* (2003) showed that major genotypic clusters linked to geographical origin could be distinguished through gel based AFLP with *O. viciifolia*.

SSR analysis is another widely used method to assess plant genotypic diversity and, more frequently than AFLP, to develop molecular markers for modern breeding programmes. Given the problems encountered using AFLP, it appeared as a promising alternative as profiles obtained by this method are multiple but simpler and should thus be less affected by the high sensivity of the capillary electrophoresis detection system. The large number of previously developed SSR detection systems for different classes of angiosperms was expected to provide a set of SSRs that could be used with *O. viciifolia*. Some of the SSR amplifications gave successful results with *O. viciifolia*, leading to clear and repeatable profiles; although it should be noted that generally more peaks than expected were detected which may be due to non-specific primer binding. As expected, some other SSRs did not transfer satisfactorily for use with *O. viciifolia* and can be discarded for future genetic studies of this species. For instance, four SSRs have been shown to produce informative profiles reflecting polymorphisms. Only combinations of several SSRs have led to robust genetic studies. In the case of this study, it was not possible to coherently segregate genetically divergent clusters. Other SSRs will be necessary to complete a genetic study and hopefully, select some molecular markers.

CHAPTER 7. PHYLOGENETIC ANALYSIS OF THE *ONOBRYCHIS* GENUS BY SEQUENCING INDIVIDUAL LOCI

Sequences from different part of the genome were analysed in order to clarify the phylogeny of the *Onobrychis* genus. This work was intended to identify and suggest different *O.* species valuable for future breeding programmes.

7.1. Amplification of several non-coding region sequences

All DNA barcode sequences (Chapter 2) were amplified from *Onobrychis sp.* DNA. Improvements in PCR protocols, mainly changes of annealing temperature, allowed the amplification of good PCR products with all primer pairs (Figure 53).

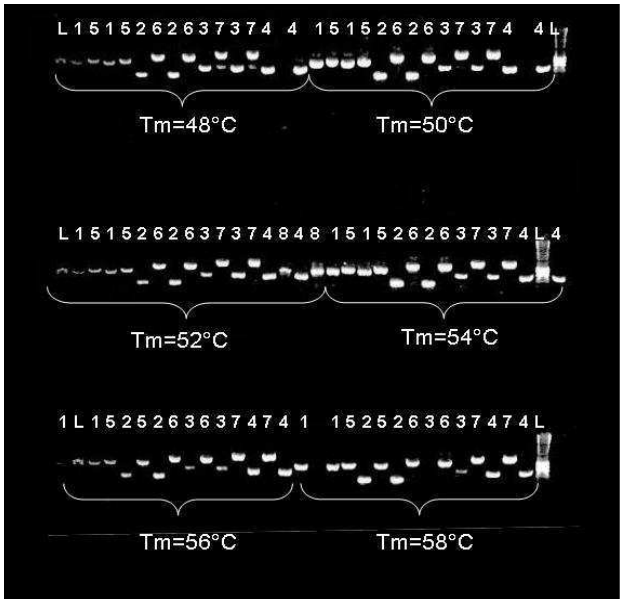

Figure 53: PCR products obtained with eight pairs of primers (1 to 8) for two different *Onobrychis viciifolia* accessions (1001 and 1256) at different annealing temperatures (48°C, 50°C, 52°C, 54°C, 56°C and 58°C)
Numbers above correspond to the primer pairs (1= psbA-trnH, 2= trnV-atpE, 3= trnC-ycf6, 4= ycf6-psbM, 5= psbM-trnD, 6= atpB-rbcL, 7= rbcL and 8= ITS), L stands for ladder (Hyperladder I from Bioline)

All regions amplified well and the optimal annealing temperatures as well as the approximate fragment sizes are reported below (Table 38).

Table 38: Region sequenced with optimal annealing temperature and approximate size of the amplified fragment

Region	**Optimal annealing temperature (°C)**	**Approximate size of fragment (bp)**
psbA-trnH	56	400
trnV-atpE	54	2000
trnC-ycf6	58	500
Ycf6-psbM	52	1500
psbM-trnD	50	600
atpB-rbcL	56	1000
rbcL	56	1000
ITS	52	600
trnT-trnL	50	500

Three regions were chosen for further sequencing. The ITS region (encompassing ITS1, the 5.8S and ITS2) and the PsbA intergenic spacer between *trnH* and *PsbA* gene were chosen as they were shown to possess a lot of variability in sequence (Kress *et al.*, 2005). Another non-coding chloroplastic DNA region, the cd intergenic spacer between *trnT* and the 5' exon of *trnL* was also sequenced, as it was successfully used in a pilot experiment. For all primer pairs, amplicons of the expected size were obtained and are shown on Figure 54 (cd region, circa 460bp), Figure 55 (PsbA region, circa 270bp) and Figure 56 (ITS region, circa 630bp).

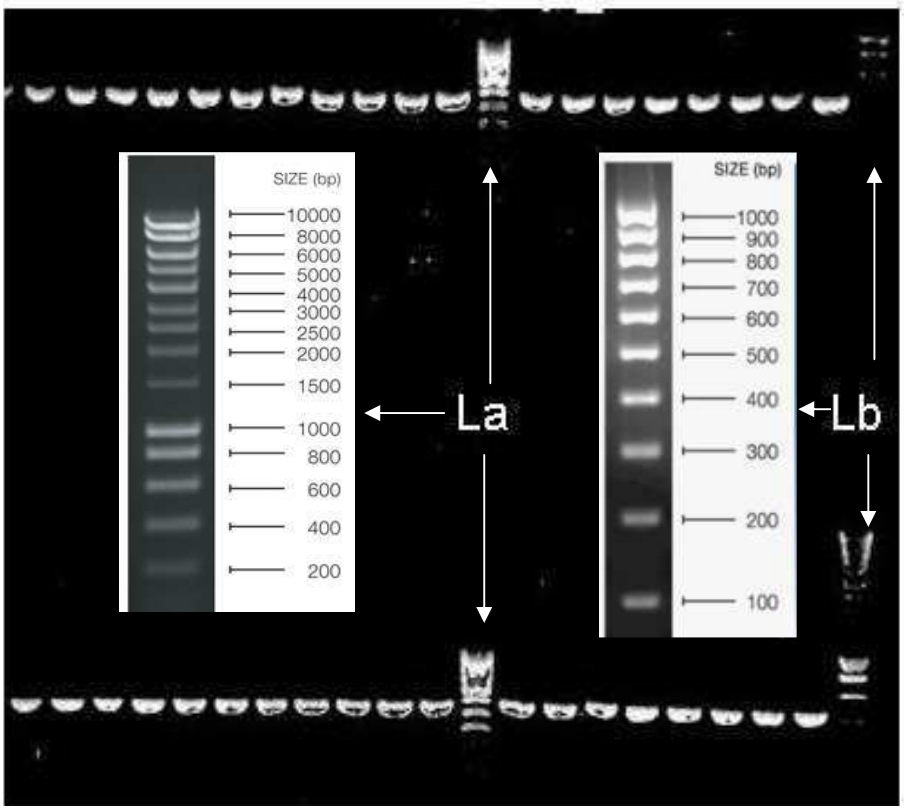

Figure 54: Example of PCR amplicons of the cd intergenic spacer between *trnT* and the 5' exon of *trnL*, La stands for Hyperladder I from Bioline and Lb stands for Hyperladder IV from Bioline

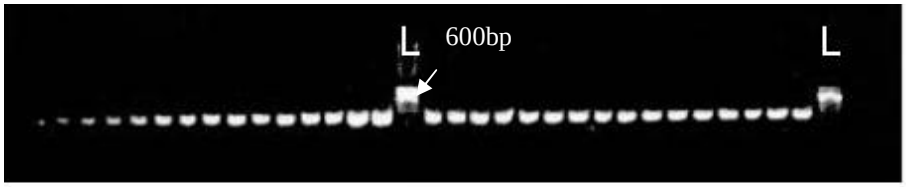

Figure 55: PCR amplicons of the PsbA intergenic spacer between *trnH* and *PsbA* gene, L stands for Hyperladder I from Bioline

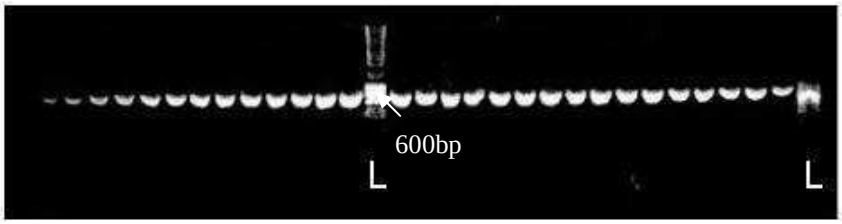

Figure 56: PCR amplicons of the Intergenic Transcribed Spacer (ITS) region, encompassing ITS1, the 5.8S and ITS2, L stands for Hyperladder I from Bioline

7.2. DNA sequences from various *Onobrychis* accessions

In total, 85 accessions were sequenced. This set of accessions was comprised of one outgroup (*Lotus corniculatus*), 29 *O. viciifolia* and 55 other *Onobrychis* sp. In some cases, sequencing was performed on different individual plants from the same accession in order to assess the genetic homogeneity of the accessions. Amplicons of the expected size were obtained for all *Onobrychis* accessions and for the *Lotus corniculatus* outgroup.

Sequences were checked for quality and aligned. The sequences for the 3 regions have been deposited in GenBank, their accession numbers are HM542483-HM542907. ITS was the most informative region (55% identical sites, 96.2% pairwise identity) followed by by PsbA and cd (77% and 78% identical sites, 98.8% pairwise identity respectively).

7.3. Phylogenetic affiliations

Phylogenetic trees were built with the DNAdist and neighbour joining (500 bootstraps) method in Phylip. Sequences from the three regions were merged in order to maximise the phylogenetic information (approximately 1360 bp in total).

The phylogenetic tree that was obtained supports the distinction of different clades corresponding to different sections among the *Onobrychis* genus (Figure 57). Heliobrychis and Hymenobrychis sections were clearly clustered together respectively.

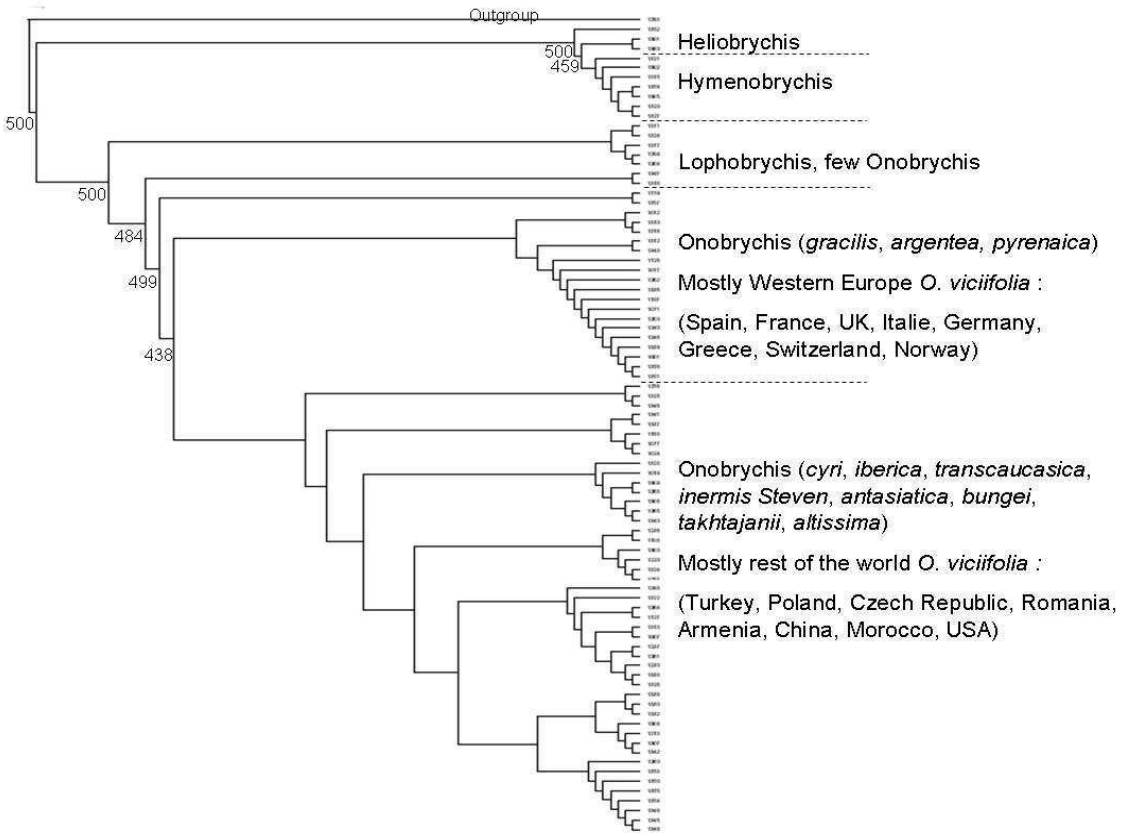

Figure 57: General neighbour joining tree based on merged Intergenic Transcribed Spacer / intergenic spacer between *trnH* and *PsbA* gene/ intergenic spacer between *trnT* and the 5' exon of *trnL* regions showing general distinctions according to sections and geographic origin in the *Onobrychis* genus. Bootstrap values (out of 500) are shown underneath selected nodes

The Lophobrychis cluster was less coherent; it was comprised of one diploid *O. viciifolia* (which might have been misidentified) and one *O. petraea* (Figure 58). As expected, the Onobrychis section was comprised of most of the accessions in the tree. In this large group, a general distinction was found between accessions from Western Europe and accessions from the rest of the world. Western European *O. viciifolia* were clustered with *O. gracilis*, *O. argentea*, and *O. pyrenaica* (Figure 58).

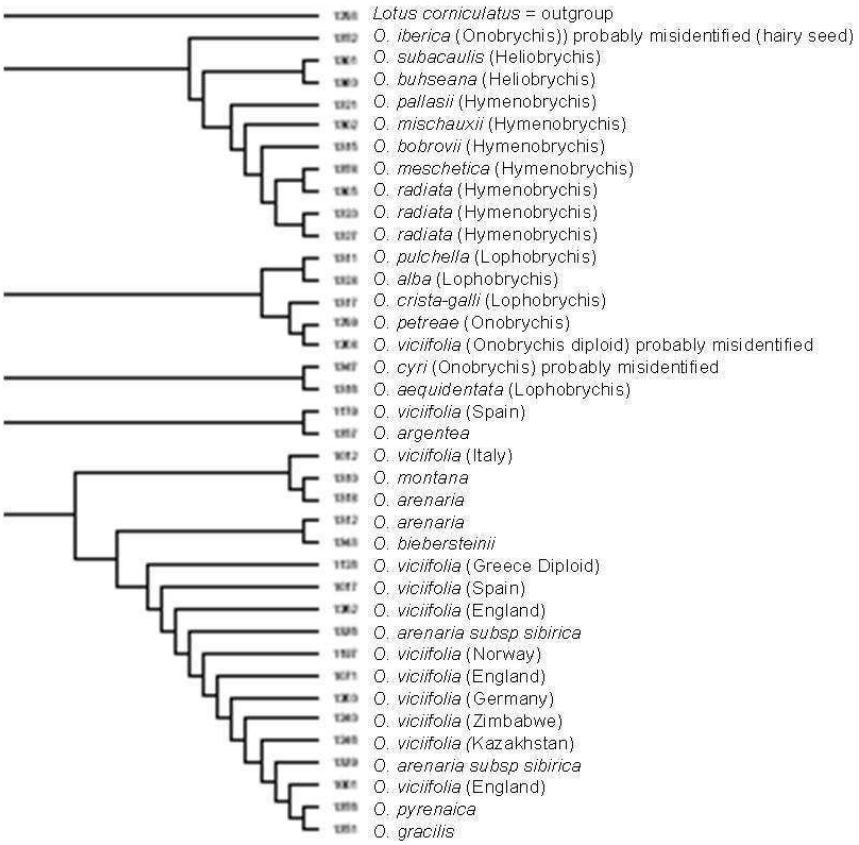

Figure 58: Upper half of the general phylogenetic tree (Figure 57) showing the Heliobrychis, Hymenobrychis, Lophobrychis and part of the Onobrychis sections as well as the Western European *O. viciifolia*.

Other *O. viciifolia* accessions were clustered with *O. cyri*, *O. transcaucasica*, *O. inermis* Steven, *O. antasiatica*, *O. bungei*, *O. takhtajanii* and *O. altissima* (Figure 59).

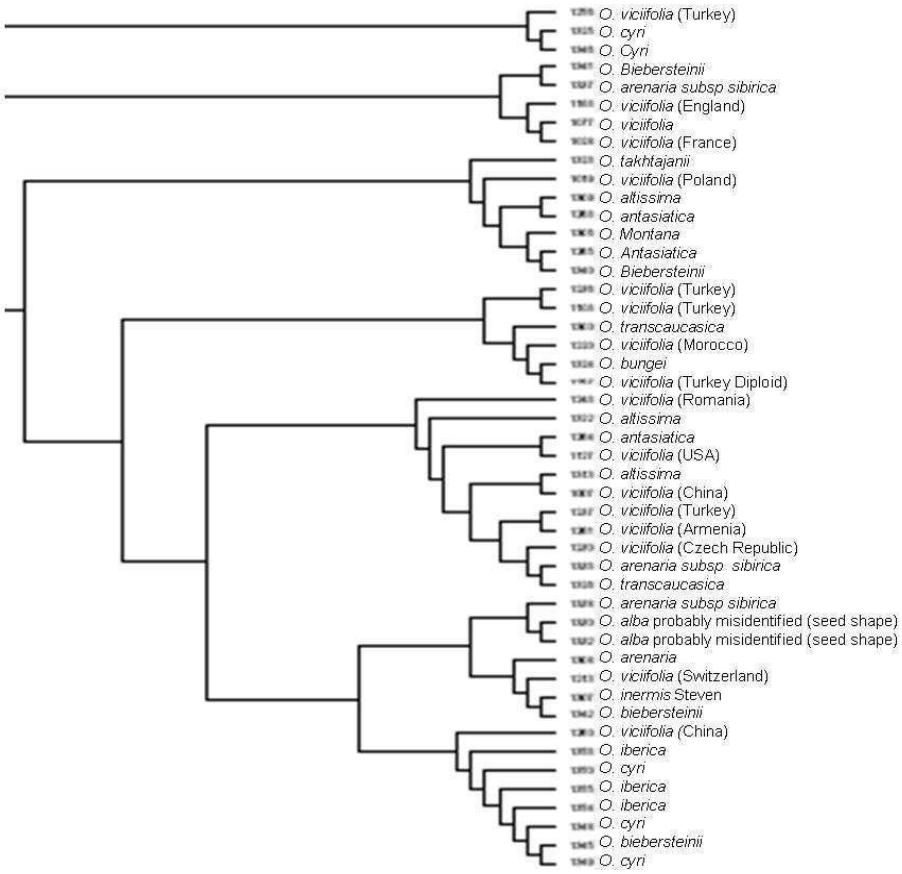

Figure 59: Lower half of the general phylogenetic tree (Figure 57) showing the part of the Onobrychis section comprising the Eastern European *O. viciifolia*.

7.4. Phylogenetic robustness of the *Onobrychis* botanical classification

Sequences obtained by other research units were added for a more global perspective.

7.4.1. Incorporation of other Hedysareae sequences

ITS sequences for other Onobrychis and members of the Hedysareae tribe were available in GenBank (Ahangarian, 2007; Chennaoui-Kourda *et al.*, 2007). A phylogenetic tree including these sequences, in addition to the ITS sequences obtained for the diverse *Onobrychis* accessions was thus built with the DNAdist and neighbour joining (500 bootstraps) method in Phylip (Figure 60).

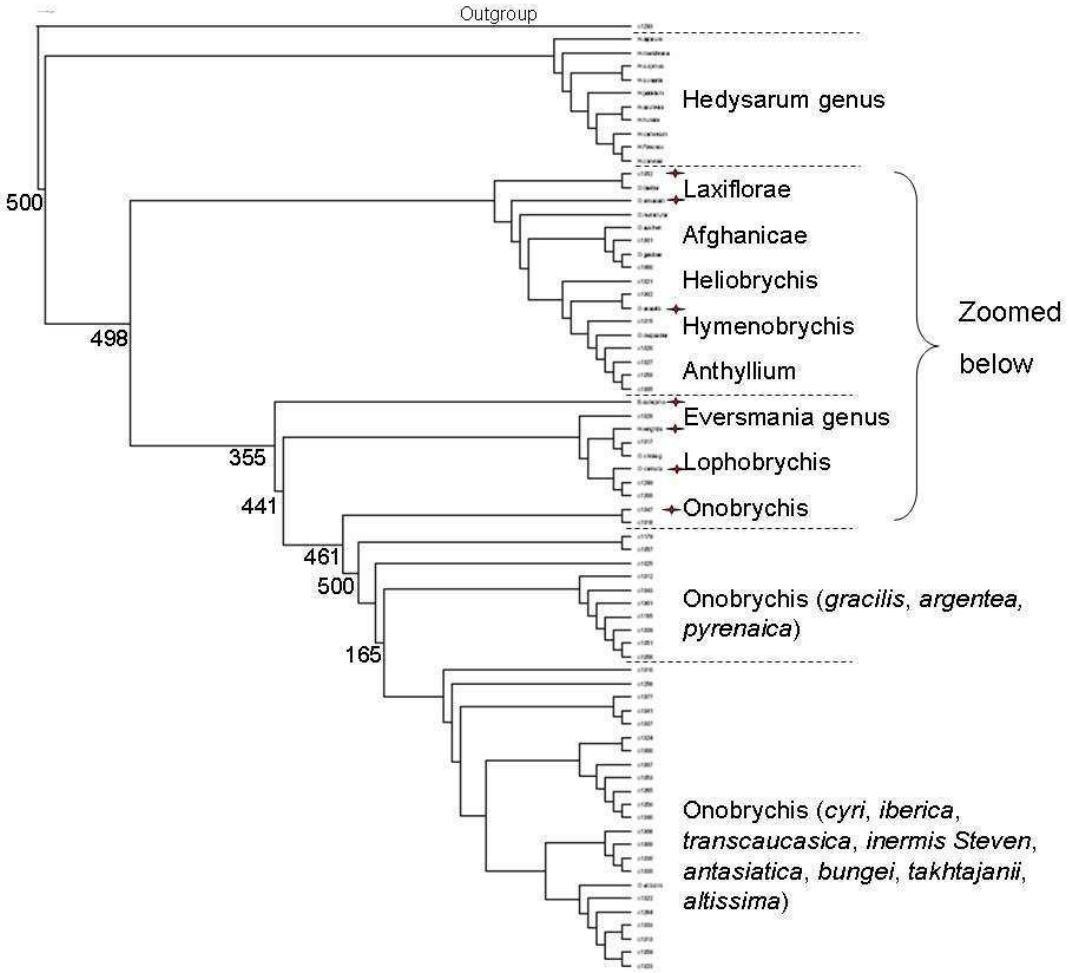

Figure 60: Neighbour joining tree based on Intergenic Transcribed Spacer sequences of selected *Onobrychis* accessions and some Hedysareae member sequences available on GenBank. Bootstrap values (out of 500) are shown underneath selected nodes

Members of the other genus were segregated from the *Onobrychis* genus, except for two species (Figure 61). *Eversmania subspinosa* was found to be more closely related to the Hymenobrychis and Lophobrychis sections. *Hedysarum wrightianum* was found to cluster with the Lophobrychis section. Some sections appeared weakly supported by the ITS phylogeny. *Onobrychis acaulis*, a member of the Anthyllium section was found to be closely related to the Hymenobrychis section. *O. cornuta*, member of the Dendrobrychis was found closely related to the Lophobrychis section.

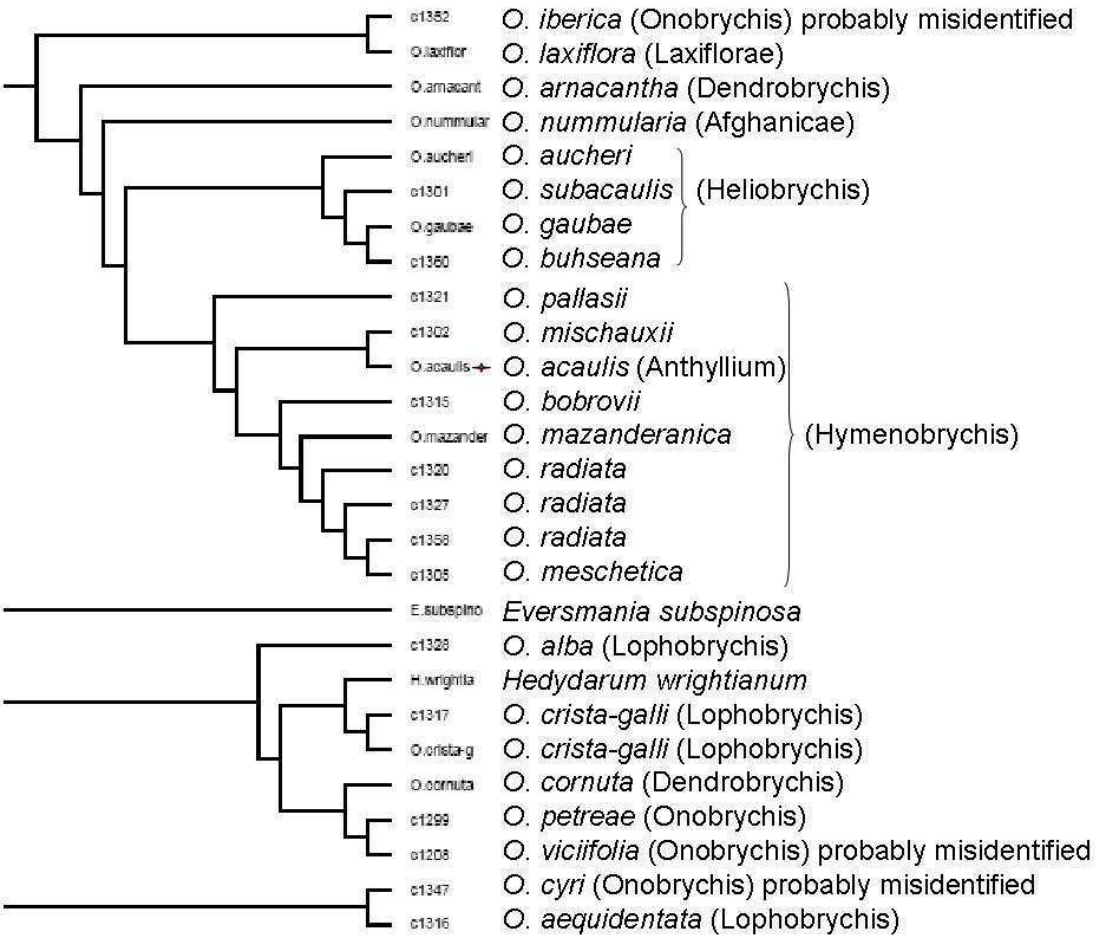

Figure 61: Part of the phylogenetic tree (Figure 60) based on Intergenic Transcribed Spacer sequences showing the different sections (Onobrychis, Laxiflorae, Dendrobrychis, Afghanicae, Heliobrychis, Hymenobrychis, Lophobrychis) of the *Onobrychis* genus

7.4.2. Operational taxonomic unit assignment

Operational taxonomic units (OTUs) encompassing all the *Onobrychis* accessions were defined using the same sequencing data used for phylogenetic tree construction. ITS sequence, chloroplastic sequences (psbA+cd) and all sequences merged were used to define OTUs, with different cut-offs due to differing variability in sequences. A 1% cut-off was used for ITS and all merged sequences, whereas a 0.1% cut-off was used for chloroplastic sequences. 10 OTUs were defined for all merged sequences, 14 for chloroplastic sequences and 13 for ITS sequences. Thus, all the sequences merged appeared to be a good representation of the OTU richness

in this study. It should be noted that, in this case, 7 OTUs were represented by one or two sequences.

7.4.3. Coherence of OTUs and botanical species/sections

In order to test the coherence of the botanical classification compared to the DNA phylogeny, OTU affiliation was compared with botanical species and section affiliation. A first screening confirmed the presence of many synonyms and subspecies in the botanical classification. Thus *O. pyrenaica*, *O. altissima*, *O. arenaria*, *O. inermis*, *O. montana* and O. *cadmea* were all considered as being *O. viciifolia*. *O. pulchella* was considered as *O. alba*, and *O. antasiatica* as *O. transcaucasica*. These synonymies were in accordance with the phylogenetic OTU affiliation for each set of sequences data.

Figures 62 (ITS and chloroplastic sequences), 63 (chloroplastic sequences) and 64 (ITS sequences) show the OTU affiliation according to section. Only the Heliobrychis section consists of a single OTU when considering all merging options (Figures 62, 63 and 64). The Hymenobrychis section is split in 2 OTUs based on ITS or on chloroplastic sequences, but only one based on all sequences merged together. The data indicate that the Lophobrychis section is much more diverse with 4 or 5 OTUs. The Onobrychis section is mainly split in two major OTUs, which probably reflects the distinction between Eastern and Western European shown by the phylogenetic trees.

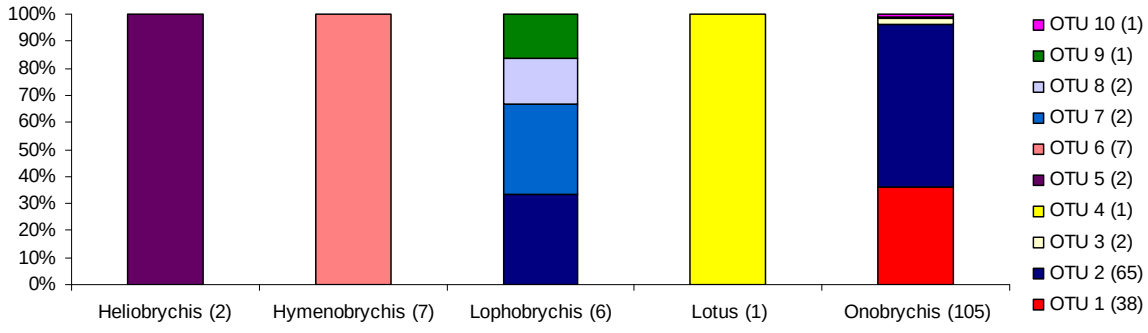

Figure 62: Operational Taxonomic Unit affiliation according to section based on all sequences merged together (ITS and chloroplastic regions *psbA-trnH* and the intergenic spacer between *trnT* and the 5' exon of *trnL*). Numbers in brackets represent the number of individuals

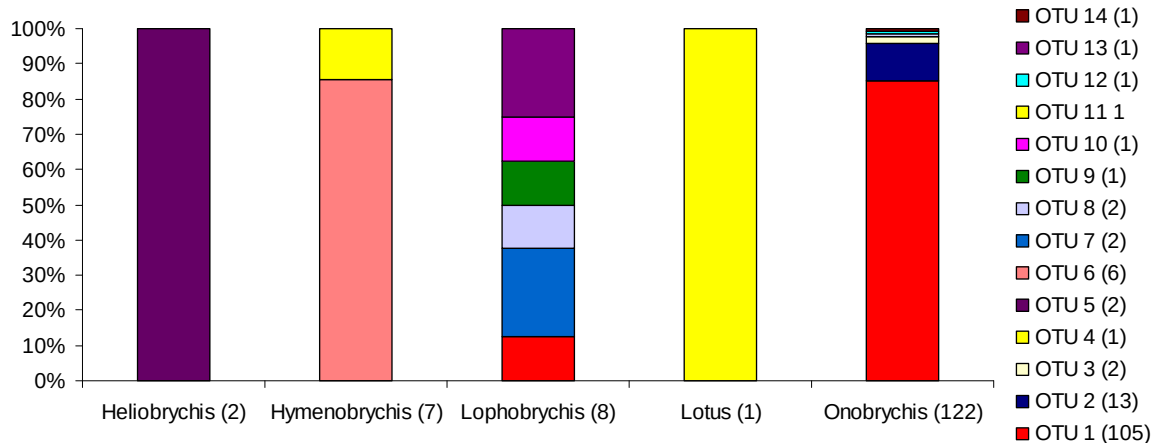

Figure 63: Operational Taxonomic Unit affiliation according to section based on chloroplastic regions (*psbA-trnH* and the intergenic spacer between *trnT* and the 5' exon of *trnL*) sequences. Numbers in brackets represent the number of individuals

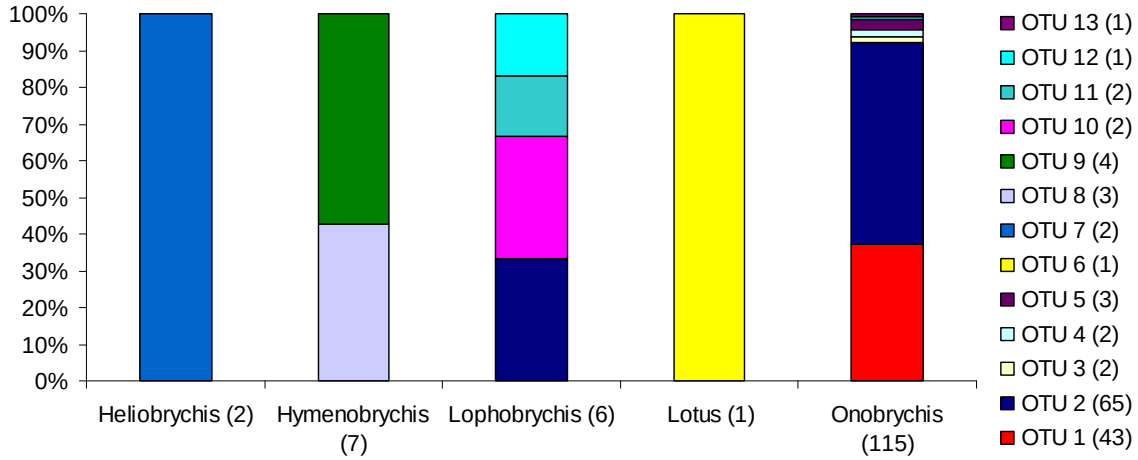

Figure 64: Operational Taxonomic Unit affiliation according to section based on ITS sequences. Numbers in brackets represent the number of individuals

Interestingly, the ratios of each of these OTUs appear to change according to the region analysed.

Figure 65 shows the OTU affiliation according to the species affiliation. Out of the 8 species represented by multiple accessions, 5 are split between 2 or 3 OTUs. Among the members of the Onobrychis section, only *O. petraea*, one *O. cyri*, and one *O. iberica* are significantly different to the rest segregated in 2 OTUs.

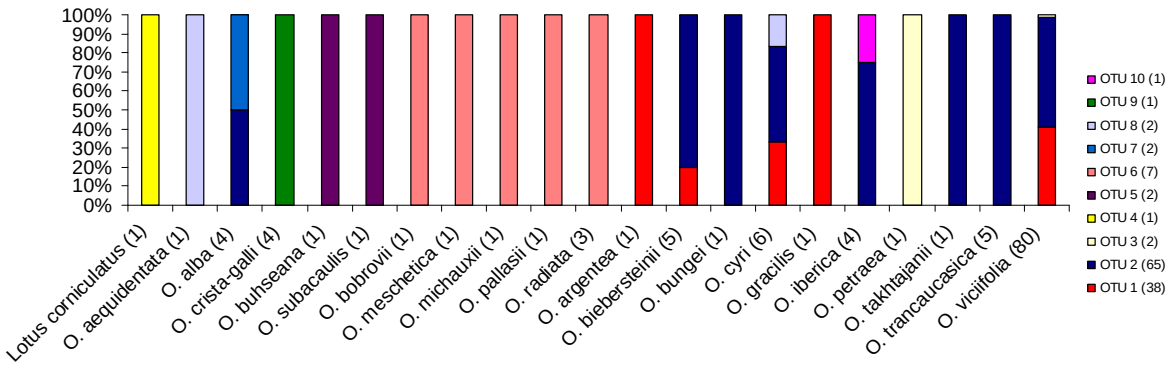

Figure 65: Operational Taxonomic Unit affiliation according to species based on all sequences merged together (ITS and chloroplastic regions psbA-trnH and the intergenic spacer between trnT and the 5' exon of trnL). Numbers in brackets represent the number of individuals

It appeared that one *O. iberica* accession has probably been misidentified based on drastic differences in seed pod morphology (Figure 66).

Figure 66: Comparison of *O. iberica* seed pod morphology showing that accession 1352 was probably misidentified (drastic differences in seed pod size and shape) compared to 1354, a standard *O. iberica* accession showing traditionally shaped fruits

O. alba is the only species from the other sections to be represented by 2 OTUs, but there might also have been misidentification based on differences in seed pod morphology (Figure 67).

Figure 67: Comparison of *O. alba* seed pod morphology showing that accession 1332 was probably misidentified (drastic differences in seed pod size and shape) compared with 1328 standard accession of *O. alba* showing traditionally shaped fruits. Accession 1330 was represented by true seeds so shape could not be compared

7.5. Discussion

PCR primers designed for various phylogenetic studies of plants, mainly legumes, were successfully used for amplification from various regions of *Onobrychis* DNA. This shows that *Onobrychis* shares genetic similarities with related plant genera. This was expected given botanical and morphological similarities with these other genera.

Among *O. viciifolia* accessions, a general phylogenetic distinction was

observed based on different DNA regions and phylogenetic methods. Similar to the agronomical and morphological analyses, it appears that this distinction is strongly connected to each accession's geographic origin. The data indicates that accessions from Western European countries are distinct from assessions from the rest of the world, forming two different clades/OTUs. This distinction may reflect different domestication routes, with different common ancestors. It can also be due to selection of various agronomic properties that would be genetically determined. The apparent agronomic superiority of the Eastern European accessions implies that this phylogenetic group and its associated genetic characteristics may be of great interest for modern breeding programmes.

The *Onobrychis* taxonomy found in the literature is relatively complex, and little information on its basis is available. For the *Onobrychis* species studied by sequencing, no less than seven sections are described. It is possible to find at least 40 species names associated with the *Onobrychis* genus. However, many synonyms and subspecies are probably over-complicating this taxonomic classification. For example, among this germplasm collection, 7 species name were used to describe different accessions that were actually all *O. viciifolia*. The molecular analysis performed can thus greatly clarify this classification. The Heliobrychis and the Hymenobrychis sections comprise very closely related species, thus it might be that the section concept is meaningless and that these sections correspond to single clearly different species. The Lophobrychis section comprises different OTUs, which support the taxonomic classification. The Onobrychis section is mainly composed of the 2 *O. viciifolia* OTUs. From the seed morphology, it appears that the *O. cyri* and the *O. iberica* accessions that belonged different OTUs were probably misidentified. In this section, only *O. petraea* appears to genetically differ from the other species. The definition of the other species is then questionable from a molecular point of view, given that all species from the Onobrychis section are very similar in morphology to *O. viciifolia*.

Sequences obtained from other species also appear to question the taxonomic classification. The Dendrobrychis section does not appear to be monophyletic. Rather, it seems than one of its members, *O. cornuta*, could be associated with the Lophobrychis section, which would leave *O. arnacantha* as a lonely different species. Similarly, *O. acaulis*, which is regarded as a member of the Anthyllium,

appears to cluster closely with the Hymenobrychis section. *Eversmania subspinosa* and *Hedysarum wrightianum* were found to be more closely related to *Onobrychis* species than to their suppose relatives (Hedysareae). Particularly, *H. wrightianum* fell in the *Lophobrychis* section with high similarity in ITS sequences with *O. crista-galli*. Again, this might be due either to misidentification of the *H. wrightianum* accession, or to a flaw in the general taxonomic classification of the Hedysareae tribe.

There is a general difficulty in coherent segregation of the Lophobrychis and Onobrychis sections. *O. petraea*, member of the Onobrychis section, is more closely related to the Lophobrychis section, as are some accessions described (but maybe misidentified) as *O. viciifolia*. It appears possible that these two sections have a relatively recent common ancestor and that one of these sections is derived from the other. These phylogenetic studies tend to show that the *Onobrychis* and probably Hedysaraea classifications generally lack genetic support. Assignations to sections, species or sub-species seem to have often been based on subjective factors (geographic origin, for example, with *O. antasiatica* and *O. transcaucasica*) rather than objective ones. As the phenotypic classification is unclear, it is difficult to extrapolate on the classification from molecular data. It is however unequivocal, that some taxonomic species or sections are too similar in their genetic content to support such a distinction. A combination of these phylogenetic analyses with morphological taxonomy and reproductive tests should lead to a simpler and more coherent classification of the *Onobrychis* genus. Such a classification is needed to improve the potential integration of species other than *O. viciifolia* into breeding programmes.

CHAPTER 8. GENERAL DISCUSSION

To my knowledge, this study represents the first characterisation of such an extensive *Onobrychis* germplasm. The diversity in geographic and climatic origins, in cultivation status and the inclusion of species different to *O. viciifolia* provides an immense database. Links with beneficial properties will furthermore open new perspectives for modern breeding and agricultural use in the context of sustainable agriculture.

8.1. Agronomical diversity and potential

The germplasm was found to be highly variable in terms of agronomic performance and characteristics. In addition, it was found that different characteristics might be selected for different agricultural uses. A streamlined scoring approach was often applied to overcome time delays that would have arisen from replicated and more precise numerical measurements. It was also a way to try to describe accession traits as a whole by reducing the weight of intra-accession variability that is often observed.

The DUS characterisation approach is a widely accepted standard approach used in modern breeding programmes. There are template frames for a variety of plant species, generally of high agricultural importance. As *O. viciifolia* has not been of such importance in the past 50 years, in Western countries at least, the DUS template that is available has not been very well developed or updated. Thus the improvements in agronomical and morphological characterisation and the results found in this study must greatly help in future development of a new DUS protocol for *O. viciifolia*.

As stated in previous literature, *O. viciifolia* was found to be relatively resistant to diseases and pests. Thus persistence was satisfactory, despite the occurrence of some periods of field flooding during the course of the project that some authors have suggested would eliminate most plants.

Strong links were found between geographic origin and accession performance. Interestingly, the best agronomical accessions mainly originate from

Eastern European countries, where it is likely that selection has been rudimentary. It can therefore be expected that modern breeding programmes will result in development of high performance varieties, which would represent a more sustainable alternative to the forage crops currently intensively cultivated.

8.2. Morphological trait characterisation

The germplasm was found to be highly variable in terms of morphological characteristics. This diversity in morphological traits may be used to develop improved varieties for contrasting agricultural uses (hay/silage vs pasture). Critical adaptations were observed with accessions from mountainous regions. Such accessions may then be favoured for *O. viciifolia* cultivation in dry elevated pastures.

The general molecular distinction between Western European accessions and accessions from the rest of the world was confirmed by morphological traits. This distinction shows that accessions are adapted to contrasting climates, and that selection of relevant accessions will be crucial in order to produce locally adapted improved varieties.

8.3. Cytological aspects of *O. viciifolia*

Little and controversial information was available concerning *Onobrychis* cytology and ploidy levels. It was known (Tamas, 2006) that members of this genus are characterised by very small chromosome size, difficult to investigate by microscopic analyses. Difficulties in classical karyotyping may explain the controversy concerning the basic number of chromosomes and the ploidy level determination. The ploidy determination method based on flow cytometry is probably more powerful as it is not dependent on a precise chromosome count but on evaluation of global DNA content. It was used with *Onobrychis* for the first time and it provided crucial information for further molecular analyses. It also led to the determination of the *Onobrychis viciifolia* C-value of DNA content, another important value for molecular analyses.

Only three wild accessions of *O. viciifolia* were found to be diploid, all the

others being tetraploid. This observation strongly suggests that tetraploids were generally selected for agricultural uses, probably due to better agronomic performance. Among the other *Onobrychis* species, roughly half were found to be diploid and the other half tetraploid. The basic numbers of chromosomes was generally 7, with few occurrences of a basic number of 8. It is difficult to draw conclusions, but it is generally thought that aneuploidy events occurred in the history of *Onobrychis*, which would suggest that species harboring a basic number of 8 chromosomes might be living ancestors of the modern *Onobrychis* species.

8.4. Genotypic diversity and phylogeny

This study has provided a first largescale analysis of *O. viciifolia* and *Onobrychis* genetic diversity. In contrast to the majority of plants used in agriculture, *Onobrychis* taxonomy is only based on morphological characterisation. However, molecular methods have often provided robust and detailed taxonomic information. Investigations through various plant databases have shown that many species and sub-species distinctions were probably meaningless and affected by subjective factors.

This study represents the first attempt to carry out a molecular based clarification of the *Onobrychis* genus. AFLP analysis had previously been applied to a limited number of *Onobrychis* species. Here, the potential of AFLP analysis was confirmed but methodological improvements are needed to increase their discriminatory power. SSRs were examined for the first time and shown to be of high potential. Phylogenetic analysis based on direct sequencing of different DNA regions has also been successful and, as expected, was the more powerful method in taxonomy clarification.

There was no previous work on *Onobrychis* genetic diversity based on fingerprinting methods. Thus, any slight genetic variability was not previously detected, and development of marker assisted breeding programs has not been achieved. In this study, it was shown that classic fingerprinting methodologies could be applied to investigate *Onobrychis* genetics. As could be expected, several methodological improvements were needed to obtain accurate and solid results. Due to the other priorities inherent to this project, these methodologies have been

attempted relatively slowly, and solid data was not obtained. Still, it was shown that these methodologies have the power to uncover *Onobrychis* genotypic diversity and in the longer term, to provide potential molecular markers that could be linked to phenotypic properties. The preliminary results obtained here suggest a substantial genetic diversity across the *Onobrychis viciifolia* accessions that were studied.

The genetic diversity suggested by fingerprinting methods was confirmed by phylogenetic studies of different genomic non-coding regions. A general trend was found with two general clusters, grouping Western European accessions in one cluster and accessions from the rest of the world (mainly Eastern Europe and the Middle East) in the other. These two clusters were found to form coherent OTUs and may thus be classified as genetic subspecies. Many subclusters, with smaller genetic distances, were also found and may be classified as genetic varieties. These subclusters were not found to be related to specific geographic regions.

Onobrychis taxonomy was, to date, only based on botanical observations which probably led to subjective biases. It is clear that many species have been named after geographic locations, but that they were probably synonyms. Identical sequences were found for species with different names, confirming that the *Onobrychis* taxonomy can be simplified. In addition, the representatives of two taxonomic sections were found to have very similar or identical sequences, which subsequently questions the meaning of such sections.

8.5. Insights of beneficial properties

The present study allowed the determination of agronomical, morphological and genetic diversity among *O. viciifolia* and other species. The overall aim of the Healthy Hay project was to target promising accessions for a potential combination of agronomical traits with *O. viciifolia* evaluated beneficial properties. Diverse studies have been conducted by Healthy Hay partners on the selected accessions (unpublished data). In all cases, it was found that some accessions performed better than others. A summary of preliminary results for promising accessions is shown in Table 39. For example, accession 1043 was found to have a high anthelmintic effect and to lead to reduced methane production by cows. Accessions 1165, 1169 and 1256 were shown to possess a high content of condensed tannins and to have higher

anthelmintic effect, which may confirm the supposed link between condensed tannin richness and anthelmintic effect. Among the best agronomic varieties, accession 1019 was found to induce low methane emissions, and accession 1213 to possess high contents of condensed tannins and flavonols as well as nitrogen.

8.6. Conclusions and perspectives

As the experiments were conducted only in one geographic location, it is clear that the conclusions on agronomic superiority only apply to these environmental conditions. Then, these conclusions could also apply to a part of Western Europe, but it is not possible to extrapolate on accessions agronomic potential under different environmental conditions. A subset of the germplasm has been tested in Spain, so a comparative analysis of the shared accessions should provides some answers concerning the climate influence on agronomic performance.

The morphological observations would have gained to be conducted using a more systematic approach. There are some methodologies available that allow automated morphometric measurements of high numbers of plant samples. However, such methodologies were not easily accessible and the necessary adjustments for a new species with distinct characteristics would have been too time-consuming in the context of this project. Nonetheless, the data collected here provide a basis to develop automated morphometry for *O. viciifolia*.

It was decided to focus on *O. viciifolia* for cytological characterisation as such information was crucial to initiate genetic studies. Cytological characterisation of other *Onobrychis* species is desirable to clarify cytology and ploidy statuses of the genus and to enable genetic characterisation of species with breeding potential. The optimisation of cytological methods for *O. viciifolia* developed in this study should improve the ability to characterise extensively and systematically *Onobrychis* accessions.

If time had allowed so, a greater number of SSR would have been applied to the accessions. A more systematic approach should have led to robust conclusions, as obtained from a variety of plants. Another approach which arose during the course of these studies is the use of high throughput sequencing to detect numerous putative

markers (Schafleitner *et al.*, 2010). Such an approach would have been ideal to develop extensive molecular tools and unravel *Onobrychis* genomics; however such approaches are also very costly and more evidences of *Onobrychis* agronomic potential are probably needed before envisioning this approach.

The *Onobrychis* taxonomy is clearly challenged by the genetic data, but a more systematic approach will be needed in order to obtain a solid classification. Morphological, cytological and genetic characterisation must be performed simultaneously and repeated on different accessions to allow complete comparisons. Again, the definition of an extensive collection of molecular markers would also help to test the coherence of taxonomic groups.

Characteristics of *Onobrychis*, and *O. viciifolia* have been extensively determined through this wide ranging germplasm study. These different results represent a large database for further modern breeding to bring back *O. viciifolia* (or other species) in the context of modern and sustainable agriculture. It is believed that the beneficial properties of *O. viciifolia* will be confirmed through the different analyses performed in the course of the Healthy Hay project. These beneficial properties, combined with the potential for *O. viciifolia* improvement detailed in this study, should lead to the development of new breeding programmes. Such programmes will represent a valuable input for farmers willing to switch to sustainable agriculture and to use more environmentally friendly forage crops.

Table 39: List of promising *O. viciifolia* accessions with interesting agronomical, chemical, anthelmintical and/or environmental properties for future breeding programme; + means top ranked, - means bottom ranked

Accession	Agronomy (NIAB)	Flavonol content (Technical University of Munich)	Condenced tannin content (University of Reading)	Nitrogen content (University of Reading)	Low methane emission (Wageningen University)	Anthelmintic properties against *Haemonchus contortus* (INRA Toulouse)	Anthelmintic properties against *Ostergia ostertagi* and *Cooperia oncophora* (Royal Veterinary and Agricultural University of Denmark)
1001	+	+					
1007	+			-		-	
1012		+				-	
1013			+				-
1019	+			-	+		
1026					+		
1028			-	+	+	-	
1041				-	+	-	
1043					+	+	
1071		+		+			
1077			-		+	-	

1103					+		
1123							+
1127							+
1157		+			-		
1163		-					+
1165	-		+	+	-	+	-
1169		+	+		-	+	
1179	-					+	
1197						+	
1200				+		+	+
1210		+		+			
1213	+	+	+	+		-	
1220	-	+	-	+			
1230	+						
1256	-	-	+			+	
1260			-		+	-	
1261			-	+		-	
1262			+	+			-
1264		-	-	+			

REFERENCES

Abou-El-Enain, M.M. (2002). Chromosomal criteria and their phylogenetic implications in the genus *Onobrychis* Mill. sect. Lophobrychis (Leguminosae), with special reference to Egyptian species. *Botanical Journal of the Linnean Society* **139**, 409-414.

Ahangarian, S., Kazempour Osaloo, Sh., Maassoumi, A. A. (2007). Molecular phylogeny of the tribe Hedysareae with special reference to *Onobrychis* (Fabaceae) as inferred from nrDNA ITS sequences. *Iranian Journal of Botany* **13**, 64-74.

Aldrich, D.T.A. (1984). Lucerne, red clover and sainfoin- herbage production. *Forage Legumes*, 126-131.

Badoux, S. (1965). Etude des caracteres morphologiques, physiologiques et agronomiques de populations d'esparcette (*Onobrychis* spp). *Recherche Agronomique Suisse* **4**, 111-190.

Baimiev, A.K., Baimiev, A.K., Gubaidullin, I.I., Kulikova, O.L. and Chemeris, A.V. (2007). Bacteria closely related to *Phyllobacterium trifolii* according to their 16S rRNA gene are discovered in the nodules of Hungarian sainfoin. *Russian Journal of Genetics* **43**, 587-590.

Barea, J.M. and Azcon-Aguilar, C. (1983). Mycorrhizas and their significance in nodulating nitrogen-fixing plants. *Advances in Agronomy* **36**, 1-54.

Barea, J.M., Azconaguilar, C. and Azcon, R. (1987). Vesicular-arbuscular mycorrhiza improve both symbiotic N2 fixation and N uptake from soil as assessed with a N15 technique under field conditions. *New Phytologist* **106**, 717-725.

Barrau, E., Fabre, N., Fouraste, I. and Hoste, H. (2005). Effect of bioactive compounds from Sainfoin (*Onobrychis viciifolia* Scop.) on the in vitro larval migration of *Haemonchus contortus*: role of tannins and flavonol glycosides. *Parasitology* **131**, 531-538.

Barry, T.N. and McNabb, W.C. (1999). The implications of condensed tannins on the nutritive value of temperate forages fed to ruminants. *British Journal of Nutrition* **81**, 263-272.

Bate-Smith, E.C. (1973). Tannins in herbaceous leguminosae. *Phytochemistry* **12**, 1809-1812.

Bland, B.F. (1971) *Crop Production: Cereals and Legumes*. London Academic Press ed. London.

Blouin, M.S., Parsons, M., Lacaille, V. and Lotz, S. (1996). Use of microsatellite loci to classify individuals by relatedness. *Molecular Ecology* **5**, 393-401.

Borreani, G., Peiretti, P.G. and Tabacco, E. (2003). Evolution of yield and quality of sainfoin (*Onobrychis viciifolia* Scop.) in the spring growth cycle. *Agronomie* **23**, 193-201.

Burton, J.C. and Curley, R.L. (1968). Nodulation and nitrogen fixation in sainfoin (*Onobrychis sativa* LAM.) as influenced by strains of rhizobia. *Sainfoin Symposium*, 3-5.

Carleton, A.E. and Wiesner, L.E. (1968). Production of sainfoin seed. *Sainfoin Symposium, Montana State University, Dec 12-13 1968*,

Cash, S.D. and Ditterline, R.L. (1996). Seed size effects on growth and N-2 fixation of juvenile sainfoin. *Field Crops Research* **46**, 145-151.

Caygill, J.C. and Mueller-Harvey, I. (1999) *Secondary Plant Products - Considerations for Animal Feeds*. Nottingham University Press ed.

Celiktas, N., Can, E., Hatipoglu, R. and Avci, S. (2006). Somatic embryogenesis, callus

production, and plantlet growth in sainfoin (*Onobrychis viciifolia* Scop.). *New Zealand Journal of Agricultural Research* **49**, 383-388.

Chen, B. (1992) *Sainfoin*. Gansun Sci-tech Press ed. Lanzhou, China.

Chennaoui-Kourda, H., Marghali, S., Marrakchi, M. and Trifi-Farah, N. (2007). Genetic diversity of Sulla genus (*Hedysareae*) and related species using Inter-simple Sequence Repeat (ISSR) markers. *Biochemical Systematics and Ecology* **35**, 682-688.

Clarke, R.T.J. and Reid, C.S.W. (1974). Foamy bloat of cattle - review. *Journal of Dairy Science* **57**, 753-785.

Cooper, C.S. (1972). Establishment, hay yield, and persistence of two sainfoin growth types seeded alone and with low growing grasses and legumes. *Agronomy Journal* **64**, 379-381.

De Giorgio, D., Maiorana, M. and Fornaro, F. (2000). Root growth analysis in sainfoin (*Onobrychis viciifolia* Scop.) submitted to different harvest times. *Legumes for Mediterranean forage crops, pastures and alternative uses : Légumineuses pour cultures fourragères, pâturages et autres usages en région méditerranéenne*, 157-160.

DeLaat, A.M.M., Gohde, W., Vogelzakg, M. J. D. C. (1987) Determination of ploidy of single plants and plant populations by flow cytometry. p. 303-307, vol. 99.

Dolezel, J., Greilhuber, J. and Suda, J. (2007). Estimation of nuclear DNA content in plants using flow cytometry. *Nature Protocols* **2**, 2233-2244.

Doyle, C.J., Thomson, D.J. and Sheehy, J.E. (1984). The future of sainfoin in british agriculture an economic assessment. *Grass and Forage Science* **39**, 43-51.

Dubbs, A.L. (1968). The performance of sainfoin and sainfoin-grass mixture on dryland in central Montana. *Sainfoin symposium; 1968 December 12-13, Bozeman, MT: Montana Agricultural Experiment Station Bulletin* **627**, 22-25.

Eken, C. (2003). First report of *Ascochyta fabae* on sainfoin in Turkey. *Plant Pathology* **52**, 421-421.

Eken, C., Demirci, E. and Dane, E. (2004). Species of *Fusarium* on sainfoin in Erzurum, Turkey. *New Zealand Journal of Agricultural Research* **47**, 261-263.

Emre, I., Turgut-balik, D., Sahin, A., Kursat, M. (2007). Total electrophoretic band patterns of some *Onobrychis* species growing in Turkey. *American-Eurasian Journal of Agricultural & Environmental Science* **2**, 123-126.

Frame, J., Charlton, J.F.L. and Laidlaw, A.S. (1998) *Temperate forage legumes*. CAB International ed. Wallingford.

Fulton, T., Chunwongse, J. and Tanksley, S. (1995). Microprep protocol for extraction of DNA from tomato and other herbaceous plants. *Plant Molecular Biology Reporter* **13**, 207-209.

Goplen, B.P., Richards, K.W. and Moyer, J.R. (1991) *Sainfoin for western Canada*. Agriculture Canada ed. Ottawa, ON.

Gray, F.A., Shigaki, T., Koch, D.W., Delaney, R.D., Gray, A.M., Majerus, M.E., Cash, D., Ditterline, R.L., Wichman, D.M. . (2006). Registration of 'Shoshone' Sainfoin. *Crop Science* **46**, 988.

Guner, A., Ozhatay, N., Ekim, T., Baser, K. (2000) Flora of Turkey and East Aegean Islands, vol. 11, pp. 98-99. Editted by Press, E.U.

Gutierrez, M.V., Vaz Patto, M.C., Huguet, T., Cubero, J.I., Moreno, M.T. and Torres, A.M. (2005). Cross-species amplification of Medicago truncatula microsatellites across three major pulse crops. *Theoretical and Applied Genetics* **110**, 1210-1217.

Harrison, M.J. (1998). Development of the arbuscular mycorrhizal symbiosis. *Current Opinion in Plant Biology* **1**, 360-365.

Hartlib, S. (1652) *His legacy*.

Hartwig, U.A. and Nosberger, J. (1996). Symbiotic nitrogen fixation: A physiological link between plant and environment. 36-43.

Heckendorn, F., Haring, D.A., Maurer, V., Senn, M. and Hertzberg, H. (2007). Individual administration of three tanniferous forage plants to lambs artificially infected with *Haemonchus contortus* and *Cooperia curticei. Veterinary Parasitology* **146**, 123-134.

Heckendorn, F., Haring, D.A., Maurer, V., Zinsstag, J., Langhans, W. and Hertzberg, H. (2006). Effect of sainfoin (*Onobrychis viciifolia*) silage and hay on established populations of *Haemonchus contortus* and *Cooperia curticei* in lambs. *Veterinary Parasitology* **142**, 293-300.

Hedqvist, H., Mueller-Harvey, I., Reed, J.D., Krueger, C.G. and Murphy, M. (2000). Characterisation of tannins and in vitro protein digestibility of several *Lotus corniculatus* varieties. *Animal Feed Science and Technology* **87**, 41-56.

Hegarty, M.J. and Hiscock, S.J. (2008). Genomic clues to the evolutionary success of polyploid plants. *Current Biology* **18**, R435-444.

Hill, R. (1998). Sainfoin: The not quite forgotten legume. *Alternative Forages for Ruminants*, 55-59.

Hoste, H., Gaillard, L. and Le Frileux, Y. (2005). Consequences of the regular distribution of sainfoin hay on gastrointestinal parasitism with nematodes and milk production in dairy goats. *Small Ruminant Research* **59**, 265-271.

Hughes, S.J. (1949). Studies on some diseases of sainfoin (*Onobrychis sativa*) II. The life history of *Ramularia onobrychidis* Allescher. *Transactions of the British Mycological Society* **32**, 34-59.

Hutchinson, J.B. (1965). Crop plant evolution: a general discussion. *Essays on Crop Plant Evolution*, 166-181.

Hwang, S.F., Berg, B.P., Howard, R.J. and Mcandrew, D.W. (1992). Screening of Sainfoin cultivars and lines for yield, winter hardiness and resistance to *Fusarium* crown and root-rot in East Central Alberta. *Canadian Plant Disease Survey* **72**, 107-111.

IUCN. (1974) Occasional Paper no. 9: Biotic provinces of the world: Further development of a system for defining and classifying natural regions for purposes of conservation, International Union for Conservation of Nature and Natural Resources, Morges, Switzerland.

Jabbar, A., Iqbal, Z., Kerboeuf, D., Muhammad, G., Khan, M.N. and Afaq, M. (2006). Anthelmintic resistance: The state of play revisited. *Life Sciences* **79**, 2413-2431.

Jensen, E.H. and Sharp, M.E. (1968). Agronomic evaluation of sainfoin in Nevada. *Sainfoin Symposium at Montana State University*,

Kaldy, M.S., Hanna, M.R. and Smoliak, S. (1979). Amino-acid composition of Sainfoin forage. *Grass and Forage Science* **34**, 145-148.

Karlin, S. and Mrazek, J. (1997). Compositional differences within and between eukaryotic genomes. *Proceedings of the National Academy of Sciences* **94**, 10227-10232.

Karnezos, T.P., Matches, A.G. and Brown, C.P. (1994). Spring lamb production on alfalfa, sainfoin, and wheatgrass pastures. *Agronomy Journal* **86**, 497-502.

Kidambi, S.P., Mahan, J.R. and Matches, A.G. (1990a). Interspecific variation for thermal dependence of glutathione reductase in sainfoin. *Theoretical and Applied Genetics* **79**, 600-604.

Kidambi, S.P., Mahan, J.R. and Matches, A.G. (1990b). Purification and thermal dependence of glutathione reductase from two forage legume species. *Plant*

Physiology **92**, 363-367.

Koivisto, J.M. and Lane, G.P.F. (2001) *Sainfoin. Worth Another Look*. Royal Agricultural College ed.

Koter, Z. (1965). Nutrition of leguminous plants. 1. The effect of different forms of N on the growth and symbiotic N2-fixation by red clover and sainfoin plants. 2. The effect of increasing amount of N on the yield and N2-fixation by Sainfoin (*Onobrychis viciifolia* var. persica). *Pamielnik pulawski* **20**, 3-37.

Koupaiabyazani, M.R., Mccallum, J., Muir, A.D., Bohm, B.A., Towers, G.H.N. and Gruber, M.Y. (1993a). Developmental changes in the composition of proanthocyanidins from leaves of Sainfoin (*Onobrychis viciifolia* Scop) as determined by HPLC analysis. *Journal of Agricultural and Food Chemistry* **41**, 1066-1070.

Koupaiabyazani, M.R., Muir, A.D., Bohm, B.A., Towers, G.H.N. and Gruber, M.Y. (1993b). The proanthocyanidin polymers in some species of *Onobrychis*. *Phytochemistry* **34**, 113-117.

Kress, W.J., Wurdack, K.J., Zimmer, E.A., Weigt, L.A. and Janzen, D.H. (2005). Use of DNA barcodes to identify flowering plants. *Proceedings of the Nationall Academy of Sciences* **102**, 8369-8374.

Kumar, R. and Singh, M. (1984). Tannins - Their Adverse Role in Ruminant Nutrition. *Journal of Agricultural and Food Chemistry* **32**, 447-453.

Liu, Z. and Lane, G.P.F. (2005). The effect of sowing date and autumn management on Sainfoin (*Onobrychis viciifolia*) regrowth and yield. *Proceedings of XX International Grassland Congress*,

Liu, Z., Lane, G.P.F. and Davies, W.P. (2006). The effects of establishment method on the yield of sainfoin (*Onobrychis viciifolia*) and sainfoin-grass mixtures. *Proceedings of British Grassland Society 8th Research Conference.*,

Lowry, J.B., McSweeney, C.S. and Palmer, B. (1996). Changing perceptions of the effect of plant phenolics on nutrient supply in the ruminant. *Australian Journal of Agricultural Research* **47**, 829-842.

Majak, W., Hall, J.W. and Mccaughey, W.P. (1995). Pasture management strategies for reducing the risk of legume bloat in cattle. *Journal of Animal Science* **73**, 1493-1498.

Marais, J.P.J., Mueller-Harvey, I., Brandt, E.V. and Ferreira, D. (2000). Polyphenols, condensed tannins, and other natural products in *Onobrychis viciifolia* (Sainfoin). *Journal of Agricultural and Food Chemistry* **48**, 3440-3447.

Martiniello, P. (2005). Recurrent phenotypic selection of legumes and grass perennial forage crops using Mediterranean autochthonous germplasm. *Journal of Genetics & Breeding* **59**, 3-4.

Martiniello, P. and Ciola, A. (1994). The effect of agronomic factors on seed and forage production in perennial legumes Sainfoin (*Onobrychis viciifolia* Scop) and French honeysuckle (*Hedysarum coronarium* L). *Grass and Forage Science* **49**, 121-129.

Mathre, D. (1968). Disease in sainfoin. *Sainfoin Symposium*, 65-66.

McMahon, L.R., Majak, W., McAllister, T.A., Hall, J.W., Jones, G.A., Popp, J.D. and Cheng, K.J. (1999). Effect of sainfoin on in vitro digestion of fresh alfalfa and bloat in steers. *Canadian Journal of Animal Science* **79**, 203-212.

Meyer, D.W. (1975). Yield, regrowth and persistence of sainfoin under fertilisation. *Agronomy Journal* **67**, 439-441.

Meyer, D.W. and Badaruddin, M. (2001). Frost tolerance of ten seedling legume species at four growth stages. *Crop Science* **41**, 1838-1842.

Min, B.R., Attwood, G.T., McNabb, W.C., Molan, A.L. and Barry, T.N. (2005). The effect of condensed tannins from *Lotus corniculatus* on the proteolytic activities and

growth of rumen bacteria. *Animal Feed Science and Technology* **121**, 45-58.

Molan, A.L., Attwood, G.T., Min, B.R. and McNabb, W.C. (2001). The effect of condensed tannins from *Lotus pedunculatus* and *Lotus corniculatus* on the growth of proteolytic rumen bacteria in vitro and their possible mode of action. *Canadian Journal of Microbiology* **47**, 626-633.

Morrill, W.L., Ditterline, R.L. and Cash, S.D. (1998). Insect pests and associated root pathogens of sainfoin in western USA. *Field Crops Research* **59**, 129-134.

Mowrey, D.P. and Matches, A.G. (1991). Persistence of sainfoin under different grazing regimes. *Agronomy Journal* **83**, 714-716.

Moyer, J.R. (1985). Effect of weed control and a companion crop on alfalfa and sainfoin establishment, yields and nutrient composition. *Canadian Journal of Plant Science* **65**, 107-116.

Mueller-Harvey, I. (2006). Unravelling the conundrum of tannins in animal nutrition and health. *Journal of the Science of Food and Agriculture* **86**, 2010-2037.

Negri, V. and Cenci, C.A. (1988). Morphological characterization of natural populations of *Onobrychis viciifolia* (Leguminosae) from Central Italy. *Willdenowia* **17**, 19-31.

Negri, V., Veronesi, F., Falcinelli, M. (1987). Germplasm evaluation of Onobrychis viciifolia Scop. for agronomic traits. *Genetica Agraria*,

Newman, G. (1997) *An overview of forage legumes*. Chalcombe Publication ed. Lincoln, UK.

Olson, D.M., Dinerstein, E., Wikramanayake, E.D., Burgess, N.D., Powell, G.V.N., Underwood, E.C., D'Amico, J.A., Itoua, I., Strand, H.E., Morrison, J.C., Loucks, C.J., Allnutt, T.F., Ricketts, T.H., Kura, Y., Lamoreux, J.F., Wettengel, W.W., Hedao, P. and Kassem, K.R. (2001). Terrestrial ecoregions of the worlds: A new map of life on Earth. *Bioscience* **51**, 933-938.

Paolini, V., Prevot, F., Dorchies, P. and Hoste, H. (2005). Lack of effects of quebracho and sainfoin hay on incoming third-stage larvae of *Haemonchus contortus* in goats. *Veterinary Journal* **170**, 260-263.

Parker, R.J. and Moss, B.R. (1981). Nutritional value of Sainfoin hay compared with Alfalfa hay. *Journal of Dairy Science* **64**, 206-210.

Pavlova, D.K. and Manova, V.I. (2000). Pollen morphology of the genera Onobrychis and Hedysarum (Hedysareae, Fabaceae) in Bulgaria. *Annales Botanici Fennici*, 207-217.

Peakall, R., Gilmore, S., Keys, W., Morgante, M. and Rafalski, A. (1998). Cross-species amplification of soybean (Glycine max) simple sequence repeats (SSRs) within the genus and other legume genera: implications for the transferability of SSRs in plants. *Molecular Biology and Evolution* **15**, 1275-1287.

Peel, M.C., Finlayson, B.L. and McMahon, T.A. (2007). Updated world map of the Köppen-Geiger climate classification. *Hydrology and Earth System Sciences* **11**, 1633-1644.

Piper, C.V. (1924) *Forage plants and their culture*. New York: The Macmillan Co.

Prevost, D., Bordeleau, L.M. and Antoun, H. (1987). Symbiotic effectiveness of indigenous arctic rhizobia on temperate forage legume - Sainfoin (*Onobrychis viciifolia*). *Plant and Soil* **104**, 63-69.

Prosperi, J.M., Demarquet, F., Angevain, M. and Mansat, P. (1994). Agronomic evaluation of local varieties of Sainfoin (*Onobrychis sativa* L) collected in South-East France. *Agronomie* **14**, 285-298.

Provorov, N.A. and Tikhonovich, I.A. (2003). Genetic resources for improving nitrogen fixation in legume-rhizobia symbiosis. *Genetic Resources and Crop Evolution* **50**, 89-99.

Rochon, J.J., Doyle, C.J., Greef, J.M., Hopkins, A., Molle, G., Sitzia, M., Scholefield, D.

and Smith, C.J. (2004). Grazing legumes in Europe: a review of their status, management, benefits, research needs and future prospects. *Grass and Forage Science* **59**, 197-214.

Roistacher, C.N., Bald, J.G. and Baker, K.F. (1953). The tetrazolium test for dormancy and germinability of gladiolus cormels. *Science* **118**, 186-187.

Rumball, W. and Claydon, R.B. (2005). Germplasm release - 'G35' Sainfoin (*Onobrychis viciifolia* Scop.). *New Zealand Journal of Agricultural Research* **48**, 127-128.

Sanchez-Yelamo, M.D. (2006). Rapid characterisation of some species of the genus *Onobrychis* Miller (Leguminosae : Papilionoideae) by isozyme methods. *Seed Science and Technology* **34**, 297-306.

Savo Sardaro, M.L., Molinari, L., Albertini, E., Rosellini, D., Negri, V. and Falcinelli, M. (2003). Molecular distinctiveness of wild populations of *Poa pratensis - Lolium perenne* and *Onobrychis viciifolia Plant genetics and breeding;Nature conservation and land resources* **49**, 47-49.

Scharenberg, A., Arrigo, Y., Gutzwiller, A., Soliva, C.R., Wyss, U., Kreuzer, M. and Dohme, F. (2007a). Palatability in sheep and in vitro nutritional value of dried and ensiled sainfoin (*Onobrychis viciifolia*) birdsfoot trefoil (*Lotus corniculatus*), and chicory (*Cichorium intybus*). *Archives of Animal Nutrition* **61**, 481-496.

Scharenberg, A., Arrigo, Y., Gutzwiller, A., Wyss, U., Hess, H.D., Kreuzer, M. and Dohme, F. (2007b). Effect of feeding dehydrated and ensiled tanniferous sainfoin (*Onobrychis viciifolia*) on nitrogen and mineral digestion and metabolism of lambs. *Archives of Animal Nutrition* **61**, 390-405.

Sergeeva, A.G. (1955). The effect of lucerne and sainfoin on the water-stable structure of soils under irrigation. *Pocvovedenie* **12**, 35-42.

Shan, M.H., Singh, K.N., Kachroo, D. and Khanday, B.A. (1991). Performance of lucerne and sainfoin under different cuttings and levels of phosphorus. *Indian Journal of Agronomy* **36**, 61-66.

Shaw, A.L. (1968). The certification program for sainfoin in Montana. *Sainfoin Symposium. Montana Agriculture Experimental Station Bulletin*, 77-78.

Sheehy, J.E. and McNeill, A. (1988). Factors controlling legume nitrogen fixation and their measurement in the field. *Physiological Limitations and the Genetic Improvement of Symbiotic Nitrogen Fixation*, 87-96.

Sheehy, J.E., Minchin, F.R. and McNeill, A. (1984). Physiological principles governing the growth and development of lucerne, sainfoin and red clover. *Forage Legumes*, pp. 112-125.

Sheehy, J.E. and Popple, S.C. (1981). Photosynthesis, water relations, temperature and canopy structure as factors influencing the growth of sainfoin (*Onobrychis viciifolia* Scop.) and Lucerne (*Medicago sativa* L.). *Annals of Botany* **48**, 113-128.

Sheldrick, R., Thomson, D. and Newman, G. (1987). *Legumes for Milk and Meat*,

Sheldrick, R.D. and Thomson, D.J. (1982) *Management and Utilization of Sainfoin (Onobrychis Sativa). Information Leaflet No. 13*. Grassland Research. Institute ed. Hurley, Berkshire.

Sims, J.R., Muir, M.K. and Carleton, A.E. (1968). Evidence of ineffective rhizobia and its relation to the nitrogen nutrition of sainfoin (*Onobrychis viciaefolia*). *Sainfoin Symposium. Montana Agriculture Experimental Station Bulletin* **627**, 8-12.

Širjaev, G. (1925) *Onobrychis generis revisio critica.* Faculté des Sciences de l'Université Masaryk ed. Brno.

Skadhauge, B., Gruber, M.Y., Thomsen, K.K. and Von Wettstein, D. (1997). Leucocyanidin reductase activity and accumulation of proanthocyanidins in developing legume tissues. *American Journal of Botany* **84**, 494-503.

Sprent, J. (2003). Plant biology: Mutual sanctions. *Nature* **422**, 672-674.

Taberlet, P., Gielly, L., Pautou, G. and Bouvet, J. (1991). Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* **17**, 1105-1109.

Tamas, E. (2006). Citological aspects of the *Onobrychis* genus. *Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. Agriculture* **62**,

Thomson, J.R. (1951a). Sainfoin in its first harvest year. *Journal of the British Grassland Society* **6**, 107-117.

Thomson, J.R. (1951b). Seed studies in sainfoin. *Journal of the British Grassland Society* **6**, 147-159.

Thomson, J.R. (1952). Further seed studies in sainfoin. *Grass and Forage Science* **7**, 65-69.

Tufenkci, S., Erman, M. and Sonmez, F. (2006). Effects of phosphorus and nitrogen applications and Rhizobium inoculation on the yield and nutrient uptake of sainfoin (*Onobrychis viciifolia* L.) under irrigated conditions in Turkey. *New Zealand Journal of Agricultural Research* **49**, 101-105.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Lee, T.v.d., Hornes, M., Friters, A., Pot, J., Paleman, J., Kuiper, M. and Zabeau, M. (1995) AFLP: a new technique for DNA fingerprinting. p. 4407-4414, vol. 23.

Waghorn, G.C., Jones, W.T., Shelton, I.D. and McNabb, W.C. (1990). Condensed tannins and the nutritive value of herbage. *Proceedings of the New Zealand Grassland Association* **51**, 171-176.

Wallace, L.E. (1968). Current and potential insect problems of sainfoin in America. *Sainfoin Symposium. Montana Agriculture Experimental Station Bulletin*, 67-70.

Wang, Y., Barbieri, L.R., Berg, B.P. and McAllister, T.A. (2007). Effects of mixing sainfoin with alfalfa on ensiling, ruminal fermentation and total tract digestion of silage. *Animal Feed Science and Technology* **135**, 296-314.

Wiesner, L.E., Carleton, A.E. and Cooper, C.S. (1968). Factors affecting sainfoin seed germination and emergence. *Sainfoin Symposium. Montana Agriculture Experimental Station Bulletin*, 13-15.

Woodward, F.I., Lomas, M.R. and Kelly, C.K. (2004). Global climate and the distribution of plant biomes. *Philosophical Transactions of the Royal Society B: Biological Sciences* **359**, 1465-1476.

Yildiz, B., Ciplak, B., Aktoklu, E. (1999). Fruit morphology of sections of the genus Onobrychis Miller (Fabaceae) and its phylogenetic implications. *Israel Journal of Plant Sciences* **47**, 269-282.

Zhang, Y., Sledge, M.K. and Bouton, J.H. (2007). Genome mapping of white clover (*Trifolium repens* L.) and comparative analysis within the Trifolieae using cross-species SSR markers. *Theoretical and Applied Genetics* **114**, 1367-1378.

APPENDIX 1: GERMPLASM GATHERED AT NIAB

Accessions highlighted in grey have not been used for field trials.

Accessions in bold character were selected for further characterisation (Chapter 2).

Accession	Species	Variety/Code	Status	Source
1001	***O. viciifolia***	**Cotswold Common/NA**	**Cultivated**	**RAC**
1002	*O. viciifolia*	Visnovsky/NA	NA	RAC
1003	*O. viciifolia*	Makedonka/NA	NA	RAC
1004	*O. viciifolia*	NA/NA	NA	RAC
1005	***O. viciifolia***	**Perly/NA**	**Cultivar**	**RAC**
1006	*O. viciifolia*	Huacheng No1/NA	NA	RAC
1007	***O. viciifolia***	**NA/NA**	**NA**	**RAC**
1008	*O. viciifolia*	Somborne/NA	Cultivar	RAC
1009	*O. viciifolia*	NA/WY-PX-94	NA	RAC
1010	*O. viciifolia*	Melrose/NA	NA	RAC
1011	*O. viciifolia*	Nova/NA	NA	RAC
1012	***O. viciifolia***	**Ambra/NA**	**Cultivar**	**James Laredonde**
1013	***O. viciifolia***	**Somborne /NA**	**Cultivated**	**Westcrop**
1014	*O. viciifolia*	Emyr/NA	NA	RAC
1015	*O. viciifolia*	Wild Type/NA	NA	Dr Adem Kamalak
1016	*O. viciifolia*	Wild Type/NA	Wild	Steven Bentley
1017	***O. viciifolia***	**Teruel/NA**	**Cultivated**	**CITA**
1018	***O. viciifolia***	**Villahoz/NA**	**NA**	**CITA**
1019	***O. viciifolia***	**Taja/NA**	**Cultivar**	**Charl Le Roux**
1020	*O. viciifolia*	NA/13 T21 00001	Wild	RICP
1021	*O. viciifolia*	Visnosky/13 T21 00353	Cultivar	RICP
1022	*O. viciifolia*	NA/13 T21 00373	Wild	RICP
1023	*O. viciifolia*	NA/13 T21 00374	Wild	RICP
1024	*O. viciifolia*	NA/13 T21 00502	Wild	RICP
1025	*O. viciifolia*	NA/13 T21 00511	Wild	RICP
1026	***O. viciifolia***	**Buciansky/13 T21 00525**	**Cultivar**	**RICP**
1027	*O. viciifolia*	Perly/13 T21 00526	NA	RICP
1028	***O. viciifolia***	**Simpro/13 T21 00527**	**Cultivar**	**RICP**
1029	*O. viciifolia*	Matra/13 T21 00528	Cultivar	RICP

1030	*O. viciifolia*	Kompolti/13 T21 00529	Cultivar	RICP
1031	*O. viciifolia*	Cotswold Common/13 T21 00530	Cultivar	RICP
1032	*O. viciifolia*	Emyr/13 T21 00531	Cultivar	RICP
1033	*O. viciifolia*	Fakir/13 T21 00532	Cultivar	RICP
1034	*O. viciifolia*	NA/13 T21 00552	Wild	RICP
1035	*O. viciifolia*	NA/13 T21 00575	Wild	RICP
1036	*O. arenaria*	NA/13 T21 00576	Wild	RICP
1037	*O. viciifolia*	Bicolari/RCAT028217	NA	RCAH
1038	*O. viciifolia*	NA/RCAT028219	NA	RCAH
1039	*O. viciifolia*	Ukrainskij 2795/RCAT028241	NA	RCAH
1040	*O. viciifolia*	Buceanskij/RCAT028237	NA	RCAH
1041	***O. viciifolia***	**Camaras/RCAT028291**	**NA**	**RCAH**
1042	*O. viciifolia*	Ibaneti/RCAT028292	NA	RCAH
1043	***O. viciifolia***	**Bivolari/RCAT028294**	**NA**	**RCAH**
1044	*O. viciifolia*	NA/RCAT028437	NA	RCAH
1045	*O. viciifolia*	NA/RCAT061765	NA	RCAH
1046	*O. viciifolia*	Fizes/RCAT028250	NA	RCAH
1047	*O. viciifolia*	NA/IG 109266 IFMI 899	NA	ICARDA
1048	*O. viciifolia*	NA/IG 109729 IFMI 1294	NA	ICARDA
1049	*O. viciifolia*	NA/IG 109730 IFMI 1295	NA	ICARDA
1050	*O. viciifolia*	NA/IG 109731 IFMI 1296	NA	ICARDA
1051	*O. viciifolia*	NA/IG 109732 IFMI 1297	NA	ICARDA
1052	*O. viciifolia*	NA/IG 109733 IFMI 1298	NA	ICARDA
1053	*O. viciifolia*	NA/IG 109735 IFMI 1299	NA	ICARDA
1054	*O. viciifolia*	NA/IG 109738 IFMI 1301	NA	ICARDA
1055	*O. viciifolia*	NA/IG 109841 IFMI 1398	NA	ICARDA
1056	*O. viciifolia*	NA/IG 109842 IFMI 1399	NA	ICARDA
1057	*O. viciifolia*	NA/IG 109846 IFMI 1400	NA	ICARDA
1058	*O. viciifolia*	NA/IG 109852 IFMI 1404	NA	ICARDA
1059	*O. viciifolia*	NA/IG 109917 IFMI 1419	NA	ICARDA
1060	*O. viciifolia*	NA/IG 109923 IFMI 1422	NA	ICARDA
1061	*O. viciifolia*	NA/IG 109931 IFMI 1425	NA	ICARDA
1062	*O. viciifolia*	NA/IG 109932 IFMI 1426	NA	ICARDA
1063	*O. viciifolia*	NA/IG 109933 IFMI 1427	NA	ICARDA
1064	*O. viciifolia*	NA/IG 109934 IFMI 1428	NA	ICARDA
1065	*O. viciifolia*	NA/IG 109935 IFMI 1429	NA	ICARDA
1066	*O. viciifolia*	NA/IG 109936 IFMI 1430	NA	ICARDA

1067	*O. viciifolia*	NA/IG 109960 IFMI 1454	NA	ICARDA
1068	*O. viciifolia*	NA/IG 109961 IFMI 1455	NA	ICARDA
1069	*O. viciifolia*	NA/IG 109962 IFMI 1456	NA	ICARDA
1070	*O. viciifolia*	NA/IG 109992 IFMI 1485	NA	ICARDA
1071	***O. viciifolia***	**Hampshire Common/NA**	**Cultivated**	**Henrey Edmunds**
1072	*O. viciifolia*	Redstart /NA	Wild	Henrey Edmunds
1073	*O. viciifolia*	Cotswold Common /Am 232	Cultivar	IGER
1074	*O. viciifolia*	Aberystwyth Sanfoin /Am 359	Cultivated	IGER
1075	*O. viciifolia*	Aberystwyth Sanfoin /Am 358	Cultivated	IGER
1076	*O. viciifolia*	Aberystwyth Sanfoin /Am 360	Cultivated	IGER
1077	***O. viciifolia***	**Nova /Am 354**	**Cultivar**	**IGER**
1078	*O. viciifolia*	Aberystwyth Sanfoin /Am 361	Cultivated	IGER
1079	*O. viciifolia*	NA/Am 94	Cultivated	IGER
1080	*O. viciifolia*	NA/Am 237	Wild	IGER
1081	*O. viciifolia*	Flemingstone /Am 108	Cultivar	IGER
1082	*O. viciifolia*	Hampshire Common /Am 95	Cultivar	IGER
1083	*O. viciifolia*	Common Milled /Am 186	Cultivar	IGER
1084	*O. viciifolia*	Sparta /Am 333	Cultivar	IGER
1085	*O. viciifolia*	NA/Am 112	Cultivar	IGER
1086	*O. viciifolia*	NA/Am 111	Cultivar	IGER
1087	*O. viciifolia*	Hampshire Common /Am 99	Cultivar	IGER
1088	*O. viciifolia*	NA/Am 110	Cultivar	IGER
1089	*O. viciifolia*	English Common/English giant sainfoin/Am 97	Cultivar	IGER
1090	*O. viciifolia*	NA/Am 93	NA	IGER
1091	*O. viciifolia*	Visnovsky /Am 117	Cultivar	IGER
1092	*O. viciifolia*	NA/Am 113	Cultivar	IGER
1093	*O. viciifolia*	Common Milled /Am 184	Cultivar	IGER
1094	*O. viciifolia*	Giant Milled /Am 185	Cultivar	IGER
1095	*O. viciifolia*	NA/Am 352	NA	IGER
1096	*O. viciifolia*	Hampshire Common /Am 92	Cultivar	IGER
1097	*O. viciifolia*	Visnovsky Viccsecry /Am 104	Cultivar	IGER
1098	*O. viciifolia*	NA/Am 227	Cultivar	IGER
1099	*O. viciifolia*	Eastern Counties Giant /Am 183	Cultivar	IGER
1100	*O. viciifolia*	CPI 63748/110397	NA	GRIN

1101	*O. viciifolia*	CPI 63749/110400	NA	GRIN
1102	*O. viciifolia*	NA/110404	NA	GRIN
1103	***O. viciifolia***	**Korunga/167236**	**NA**	**GRIN**
1104	***O. viciifolia***	**NA/170582**	**NA**	**GRIN**
1105	*O. viciifolia*	NA/170583	Cultivated	GRIN
1106	*O. viciifolia*	NA/170585	Cultivated	GRIN
1107	*O. viciifolia*	NA/171725	NA	GRIN
1108	*O. viciifolia*	NA/171726	NA	GRIN
1109	*O. viciifolia*	CPI 63747/178988	Cultivated	GRIN
1110	***O. viciifolia***	**CPI 63750/182247**	**NA**	**GRIN**
1111	*O. viciifolia*	CPI 63751/186520	NA	GRIN
1112	*O. viciifolia*	CPI 63752/192993	NA	GRIN
1113	***O. viciifolia***	**CPI 63753/192994**	**NA**	**GRIN**
1114	*O. viciifolia*	CPI 63754/192995	NA	GRIN
1115	*O. viciifolia*	CPI 63755/200872	Wild	GRIN
1116	*O. viciifolia*	NA/201211	Cultivar	GRIN
1117	*O. viciifolia*	CPI 63757/201512	Cultivar	GRIN
1118	***O. viciifolia***	**CPI 63758/201865**	**NA**	**GRIN**
1119	*O. viciifolia*	CPI 63759/204594	Wild	GRIN
1120	*O. viciifolia*	CPI 63760/204595	Wild	GRIN
1121	*O. viciifolia*	CPI 63761/205200	Wild	GRIN
1122	*O. viciifolia*	CPI 63762/205201	Wild	GRIN
1123	***O. viciifolia***	**CPI 63763/205202**	**Wild**	**GRIN**
1124	*O. viciifolia*	CPI 63764/206458	Wild	GRIN
1125	*O. viciifolia*	CPI 63765/206459	Cultivated	GRIN
1126	*O. viciifolia*	CPI 63766/206577	Wild	GRIN
1127	***O. viciifolia***	**CPI 63767/212241**	**Cultivated**	**GRIN**
1128	*O. viciifolia*	CPI 63768/223389	Cultivated	GRIN
1129	*O. viciifolia*	CPI 63769/225728	NA	GRIN
1130	*O. viciifolia*	CPI 63770/227038	Cultivated	GRIN
1131	*O. viciifolia*	CPI 63771/227373	Cultivated	GRIN
1132	*O. viciifolia*	CPI 63772/228156	NA	GRIN
1133	*O. viciifolia*	CPI 63773/228289	Wild	GRIN
1134	*O. viciifolia*	NA/228352	Wild	GRIN
1135	*O. viciifolia*	CPI 63775/228402	Wild	GRIN
1136	*O. viciifolia*	CPI 63776/229612	Wild	GRIN
1137	*O. viciifolia*	CPI 63777/229613	Cultivated	GRIN

1138	*O. viciifolia*	CPI 63778/234644	Wild	GRIN
1139	*O. viciifolia*	CPI 63779/234822	Wild	GRIN
1140	***O. viciifolia***	**CPI 63780/234823**	**Wild**	**GRIN**
1141	*O. viciifolia*	CPI 63781/236486	NA	GRIN
1142	*O. viciifolia*	CPI 63782/237089	Wild	GRIN
1143	*O. viciifolia*	CPI 63783/239957	NA	GRIN
1144	*O. viciifolia*	CPI 63784/239958	NA	GRIN
1145	*O. viciifolia*	CPI 63785/239959	NA	GRIN
1146	*O. viciifolia*	CPI 63786/239960	NA	GRIN
1147	*O. viciifolia*	CPI 63787/243227	NA	GRIN
1148	*O. viciifolia*	Espers/250024	Cultivated	GRIN
1149	*O. viciifolia*	NA/251669	NA	GRIN
1150	*O. viciifolia*	CPI 63791/251840	Wild	GRIN
1151	*O. viciifolia*	CPI 63792/258767	NA	GRIN
1152	*O. viciifolia*	Reskhanii 1251/258768	Cultivar	GRIN
1153	*O. viciifolia*	Dukorastuschaia/258769	Cultivar	GRIN
1154	*O. viciifolia*	Dukorastushchii/258770	Cultivar	GRIN
1155	*O. viciifolia*	Dukorastushchii/258771	Cultivar	GRIN
1156	***O. viciifolia***	**Dukorastushchii/258772**	**Cultivar**	**GRIN**
1157	***O. viciifolia***	**Miatiletka/258773**	**Cultivar**	**GRIN**
1158	*O. viciifolia*	Severo-Kavkazckii Dvuukosnii/258774	Cultivar	GRIN
1159	*O. viciifolia*	CPI 63800/258775	NA	GRIN
1160	*O. viciifolia*	Dukorastushchii/258776	Cultivar	GRIN
1161	*O. viciifolia*	Dukorastushchii/258777	Cultivar	GRIN
1162	*O. viciifolia*	Dukorastushchii/258778	Cultivar	GRIN
1163	***O. viciifolia***	**Giant/259491**	**Cultivar**	**GRIN**
1164	*O. viciifolia*	Hampshire Common /259492	Cultivar	GRIN
1165	***O. viciifolia***	**Rees "A"/259493**	**Cultivar**	**GRIN**
1166	*O. viciifolia*	Turkish anatolian/259494	Cultivar	GRIN
1167	*O. viciifolia*	CPI 63808/263158	Cultivar	GRIN
1168	*O. viciifolia*	Ukrainsky 553/263159	Cultivar	GRIN
1169	***O. viciifolia***	**CPI 63810/273784**	**NA**	**GRIN**
1170	*O. viciifolia*	Ukrainsky 586/273785	Cultivar	GRIN
1171	***O. viciifolia***	**CPI 63812/273786**	**NA**	**GRIN**
1172	*O. viciifolia*	CPI 63813/273787	NA	GRIN
1173	*O. viciifolia*	CPI 63814/273788	NA	GRIN
1174	*O. viciifolia*	CPI 63815/273789	NA	GRIN

1175	*O. viciifolia*	CPI 63816/273790	NA	GRIN
1176	*O. viciifolia*	CPI 63817/273791	NA	GRIN
1177	*O. viciifolia*	CPI 63818/302936	NA	GRIN
1178	*O. viciifolia*	CPI 63819/302937	NA	GRIN
1179	***O. viciifolia***	**CPI 63820/302938**	**NA**	**GRIN**
1180	*O. viciifolia*	CPI 63821/302939	NA	GRIN
1181	*O. viciifolia*	Lupinella/306693	Cultivar	GRIN
1182	*O. viciifolia*	B-184/311467	NA	GRIN
1183	*O. viciifolia*	CPI 63824/311468	NA	GRIN
1184	*O. viciifolia*	CPI 63825/311469	NA	GRIN
1185	*O. viciifolia*	CPI 63826/311470	NA	GRIN
1186	*O. viciifolia*	CPI 63827/311471	NA	GRIN
1187	*O. viciifolia*	CPI 63828/313046	NA	GRIN
1188	*O. viciifolia*	CPI 63829/313047	NA	GRIN
1189	*O. viciifolia*	Ziumineck/313048	NA	GRIN
1190	*O. viciifolia*	CPI 63831/313049	NA	GRIN
1191	*O. viciifolia*	CPI 63832/313050	NA	GRIN
1192	*O. viciifolia*	CPI 63833/313051	NA	GRIN
1193	*O. viciifolia*	CPI 63834/313052	NA	GRIN
1194	*O. viciifolia*	CPI 63835/313053	NA	GRIN
1195	*O. viciifolia*	CPI 63836/313054	NA	GRIN
1196	*O. viciifolia*	CPI 63837/313055	NA	GRIN
1197	***O. viciifolia***	**CPI 63838/313056**	**NA**	**GRIN**
1198	*O. viciifolia*	Ukranian 57/57/313057	Cultivated	GRIN
1199	***O. viciifolia***	**CPI 63840/313058**	**NA**	**GRIN**
1200	***O. viciifolia***	**CPI 63841/313059**	**NA**	**GRIN**
1201	*O. viciifolia*	CPI 63842/313060	NA	GRIN
1202	*O. viciifolia*	Poltava 553/313061	Cultivated	GRIN
1203	*O. viciifolia*	Artemovsk/313062	Cultivated	GRIN
1204	*O. viciifolia*	CPI 63845/313063	NA	GRIN
1205	*O. viciifolia*	Italian/313064	Cultivated	GRIN
1206	*O. viciifolia*	Dnepropetrovsk/313065	Cultivated	GRIN
1207	*O. viciifolia*	CPI 63848/313066	NA	GRIN
1208	*O. viciifolia*	CPI 63849/314099	Wild	GRIN
1209	*O. viciifolia*	CPI 31422/316296	NA	GRIN
1210	***O. viciifolia***	**Premier/318602**	**Cultivar**	**GRIN**
1211	***O. viciifolia***	**Perly/318603**	**Cultivar**	**GRIN**

1212	*O. viciifolia*	Pologne/318604	Cultivar	GRIN
1213	***O. viciifolia***	**CPI 63854/318605**	**Cultivated**	**GRIN**
1214	*O. viciifolia*	CPI 63855/318606	Cultivated	GRIN
1215	*O. viciifolia*	CPI 63856/319058	NA	GRIN
1216	*O. viciifolia*	CPI 63857/319059	NA	GRIN
1217	*O. viciifolia*	CPI 63858/319060	NA	GRIN
1218	*O. viciifolia*	CPI 63860/319062	NA	GRIN
1219	*O. viciifolia*	CPI 63861/319713	NA	GRIN
1220	***O. viciifolia***	**247/338651**	**NA**	**GRIN**
1221	*O. viciifolia*	CPI 63863/368034	NA	GRIN
1222	*O. viciifolia*	CPI 63864/368035	NA	GRIN
1223	*O. viciifolia*	Pola/368036	Cultivar	GRIN
1224	*O. viciifolia*	Krasnodarskij/372828	Cultivar	GRIN
1225	*O. viciifolia*	Svedskij/372829	Cultivar	GRIN
1226	*O. viciifolia*	Kirgizskij/372830	Cultivar	GRIN
1227	*O. viciifolia*	Germanskij/372831	Cultivar	GRIN
1228	*O. viciifolia*	Srbskij/372832	Cultivar	GRIN
1229	*O. viciifolia*	Buciansky/372833	Cultivar	GRIN
1230	***O. viciifolia***	**Visnovsky/372834**	**Cultivar**	**GRIN**
1231	*O. viciifolia*	Bendelebener/372835	Cultivar	GRIN
1232	*O. viciifolia*	73/380948	Cultivated	GRIN
1233	*O. viciifolia*	270/380949	Cultivated	GRIN
1234	*O. viciifolia*	136/383713	Cultivated	GRIN
1235	*O. viciifolia*	183/383714	Wild	GRIN
1236	*O. viciifolia*	253/383715	Wild	GRIN
1237	*O. viciifolia*	254/383716	Wild	GRIN
1238	*O. viciifolia*	255/383717	Wild	GRIN
1239	*O. viciifolia*	710/400305	NA	GRIN
1240	*O. viciifolia*	805/400306	NA	GRIN
1241	***O. viciifolia***	**CRIC 22785/401419**	**Wild**	**GRIN**
1242	*O. viciifolia*	Octo/401467	Wild	GRIN
1243	*O. viciifolia*	Sparta /401468	Wild	GRIN
1244	*O. viciifolia*	NA/401715	Cultivated	GRIN
1245	***O. viciifolia***	**D-1738/440575**	**Wild**	**GRIN**
1246	*O. viciifolia*	D-1784/440576	Wild	GRIN
1247	*O. viciifolia*	D-1800/440577	Wild	GRIN
1248	*O. viciifolia*	Sparceto/490283	Cultivated	GRIN

1249	*O. viciifolia*	R 87/494667	Wild	GRIN
1250	*O. viciifolia*	R 98/494668	Wild	GRIN
1251	*O. viciifolia*	R 113/494669	Wild	GRIN
1252	*O. viciifolia*	AR-111/502554	Cultivated	GRIN
1253	***O. viciifolia***	**Tu86-43-03/561106**	**Cultivated**	**GRIN**
1254	*O. viciifolia*	TU86-45-01/561107	Wild	GRIN
1255	*O. viciifolia*	A-4985/568207	Wild	GRIN
1256	***O. viciifolia***	**Wkt 10/568208**	**Wild**	**GRIN**
1257	*O. viciifolia*	Wkt 9/568209	Wild	GRIN
1258	*O. viciifolia*	X910080/577670	Cultivated	GRIN
1259	***O. viciifolia***	**X93076/12992**	**Wild**	**GRIN**
1260	***O. viciifolia***	**X93234/13138**	**Wild**	**GRIN**
1261	***O. viciifolia***	**Line 107/17429**	**Cultivated**	**GRIN**
1262	***O. viciifolia***	**Cotswold Common/NA**	**Cultivated**	**Cotswold seeds**
1263	*O. antasiatica*	Akhurian-107/NA	Cultivated	NAS
1264	***O. antasiatica***	**Sisiani Local/NA**	**Cultivated**	**NAS**
1265	*O. antasiatica*	Martuni Local/NA	Cultivated	NAS
1266	***O. viciifolia***	**Esparsette/NA**	**Cultivar**	**Cotswold seeds**
1267	*O. viciifolia*	Cotswold/NA	Cultivated	CITA
1268	*O. viciifolia*	Somborne/NA	Cultivar	CITA
1269	*O. viciifolia*	Esparcette/NA	Cultivar	CITA
1270	*O. viciifolia*	Sepial/NA	Cultivar	CITA
1271	*O. viciifolia*	Ambra/NA	Cultivar	CITA
1272	*O. viciifolia*	Fakir/NA	Cultivar	CITA
1273	*O. viciifolia*	Ukrania/NA	Cultivated	CITA
1274	*O. viciifolia*	Incoronata/NA	Cultivated	CITA
1275	*O. viciifolia*	Visnovsky/NA	Cultivar	CITA
1276	*O. viciifolia*	Yubileyna/NA	Cultivated	CITA
1277	*O. viciifolia*	Korunga/NA	Cultivated	CITA
1278	*O. viciifolia*	Polonia/NA	Cultivated	CITA
1279	*O. viciifolia*	9-2/NA	Cultivated	CITA
1280	*O. viciifolia*	Reznos/NA	Cultivated	CITA
1281	*O. viciifolia*	7-1/NA	Cultivated	CITA
1282	*O. viciifolia*	Mezquita de Jarque /NA	Cultivated	CITA
1283	*O. viciifolia*	Lagueruela/NA	Cultivated	CITA
1284	*O. viciifolia*	Loarre/NA	Cultivated	CITA
1285	*O. viciifolia*	Torrrecilla de Cameros /NA	Cultivated	CITA

1286	*O. viciifolia*	Graus/NA	Cultivated	CITA
1287	*O. viciifolia*	Tartareu/NA	Cultivated	CITA
1288	*O. viciifolia*	Villahermosa del Rio /NA	Cultivated	CITA
1289	*O. viciifolia*	Wild Type/NA	Wild	Christine Hayot
1290	***O. viciifolia***	**Sepial/NA**	**Cultivar**	**Caussade semences**
1291	*O. viciifolia*	Palio/NA	Cultivar	Caussade semences
1292	*O. viciifolia*	NA/NA	NA	Caussade semences
1293	*Lotus corniculatus*	Grassland Goldie/S 2942	NA	AgResearch
1294	*Lotus corniculatus*	Leo/NA	NA	NA
1295	*Lotus corniculatus*	Ecotype/NA	NA	NA
1296	*Lotus corniculatus*	Oberhaustaadter/NA	NA	NA
1297	*Lotus pedunculatus*	Grassland Maku/ST 306	NA	AgResearch
1298	*O. hajastana*	Wild Type/NA	Wild	NAS
1299	*O. petraea*	Wild Type/NA	Wild	NAS
1300	*O. transcaucasica*	Wild Type/NA	Wild	NAS
1301	*O. subacaulis*	Wild Type/NA	Wild	NAS
1302	*O. michauxii*	Wild Type/NA	Wild	NAS
1303	*O. atropatana*	Wild Type/NA	Wild	NAS
1304	*O. viciifolia*	GS. 100/Giant sainfoin	NA	NIAB
1305	*O. radiata*	Wild Type/ ONO 66	Wild	IPK
1306	*O. montana*	Wild Type/ ONO 35	Wild	IPK
1307	*O. inermis Steven*	Wild Type/ ONO 37	Wild	IPK
1308	*O. arenaria*	Wild Type/ ONO 32	Wild	IPK
1309	*O. altissima*	Wild Type/ ONO 22	Wild	IPK
1310	*O. montana*	Wild Type/ ONO 60	Wild	IPK
1311	*O. pulchella*	Wild Type/ ONO 39	Wild	IPK
1312	*O. arenaria*	Pescanyj Ulucsennyj/ ONO 28	NA	IPK
1313	*O. altissima*	Achalkalakskij/ ONO 44	NA	IPK
1314	*O. arenaria*	Wild Type/ ONO 31	Wild	IPK
1315	*O. bobrovii*	Wild Type/ ONO 19	Wild	IPK
1316	*O. aequidentata*	Wild Type/ ONO 40	Wild	IPK
1317	*O. crista-galli*	Wild Type/ ONO 15	Wild	IPK
1318	*O. arenaria*	Ukrainskij 2795/ ONO 33	NA	IPK
1319	*O. caput-galli*	Wild Type/ ONO 14	Wild	IPK

1320	*O. radiata*	Wild Type/ ONO 64	Wild	IPK
1321	*O. pallasii*	Wild Type/ ONO 57	Wild	IPK
1322	*O. altissima*	Wild Type/NA	Wild	NAS
1323	*O. takhtajanii*	Wild Type/NA	Wild	NAS
1324	*O. bungei*	Wild Type/NA	Wild	NAS
1325	*O. cyri*	Wild Type/NA	Wild	NAS
1326	*O. transcaucasica*	Wild Type/NA	Wild	NAS
1327	*O. radiata*	Wild Type/NA	Wild	NAS
1328	*O. alba ssp. Laconica*	PI 314292/563	Wild	GRIN
1329	*O. alba ssp. Laconica*	PI 369542/NA	NA	GRIN
1330	*O. alba ssp. Laconica*	PI 642147/B96-138	Wild	GRIN
1331	*O. alba ssp. Laconica*	W6 19311/B96-113	Wild	GRIN
1332	*O. alba ssp. Laconica*	W6 19337/B96-147	Wild	GRIN
1333	*O. arenaria ssp. Sibirica*	PI 369283/1738	Wild	GRIN
1334	*O. arenaria ssp. Sibirica*	PI 371534/NA	NA	GRIN
1335	*O. arenaria ssp. Sibirica*	PI 420847/3093	NA	GRIN
1336	*O. arenaria ssp. Sibirica*	PI 539025/AJC-478	Wild	GRIN
1337	*O. arenaria ssp. Sibirica*	PI 577666/DJ-4195	Cultivated	GRIN
1338	*O. arenaria ssp. Sibirica*	W6 18193/W94041	Wild	GRIN
1339	*O. arenaria ssp. Sibirica*	W6 18202/W94052	Wild	GRIN
1340	*O. biebersteinii*	PI 227377/NA	Wild	GRIN
1341	*O. biebersteinii*	PI 284124/CPI 23313	NA	GRIN
1342	*O. biebersteinii*	PI 312931/30082	NA	GRIN
1343	*O. biebersteinii*	PI 312932/CPI 63722	NA	GRIN
1344	*O. biebersteinii*	PI 325446/S-15	Wild	GRIN
1345	*O. biebersteinii*	W6 17872/VIR 058	Wild	GRIN
1346	*O. cyri*	PI 312461/NA	NA	GRIN
1347	*O. cyri*	PI 314467/CPI 63725	Wild	GRIN
1348	*O. cyri*	PI 314468/225	Wild	GRIN
1349	*O. cyri*	W6 17811/VIR D47	Wild	GRIN
1350	*O. cyri*	W6 17878/VIR 101	Wild	GRIN
1351	*O. gracilis*	W6 19496/B96-357	Wild	GRIN
1352	*O. iberica*	PI 219602/NA	Wild	GRIN
1353	*O. iberica*	PI 312934/CPI 63729	NA	GRIN

1354	*O. iberica*	PI 314931/NA	NA	GRIN
1355	*O. iberica*	PI 315085/NA	NA	GRIN
1356	*O. pyrenaica*	Wild Type/NA	Wild	CITA
1357	*O. argentea*	Wild Type/NA	Wild	CITA
1358	*O. meschetica*	Wild Type/NA	Wild	NAS
1359	*O. cadmea*	Wild Type/NA	Wild	NAS
1360	*O. buhseana*	Wild Type/NA	Wild	NAS

APPENDIX 2: ACCESSION GEOGRAPHIC ORIGIN

75 accessions selected for seed production in 2008 are highlighted in grey:

Accession	Country	Longitude	Latitude	Biome	Koppen1	Koppen2	Koppen3
1001	UK	-2	52	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1002	NA	NA	NA	NA	NA	NA	NA
1003	NA	NA	NA	NA	NA	NA	NA
1004	China	NA	NA	NA	NA	NA	NA
1005	NA	NA	NA	NA	NA	NA	NA
1006	NA	NA	NA	NA	NA	NA	NA
1007	China	NA	NA	NA	NA	NA	NA
1008	NA	NA	NA	NA	NA	NA	NA
1009	NA	NA	NA	NA	NA	NA	NA
1010	NA	NA	NA	NA	NA	NA	NA
1011	NA	NA	NA	NA	NA	NA	NA
1012	Italy	NA	NA	NA	NA	NA	NA
1013	NA	NA	NA	NA	NA	NA	NA
1014	NA	NA	NA	NA	NA	NA	NA
1015	Turkey	NA	NA	NA	NA	NA	NA
1016	UK	0	52	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1017	Spain	-0.666667	40.666667	Mediterranean Forests, Woodlands Scrub	Arid	Steppe	Cold
1018	Spain	-3.916667	42.083333	Mediterranean Forests, Woodlands Scrub	Temperate	Without dry season	Warm summer
1019	Poland	NA	NA	NA	Cold	Without dry season	Warm summer
1020	Austria	16.0531	48.531	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1021	Czechoslovakia	NA	NA	NA	NA	NA	NA
1022	Czech republic	16.516667	49.166667	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1023	Czech republic	16.516667	49.166667	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1024	Czech republic	16.583333	49.166667	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1025	Slovakia	17.3407	48.5302	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1026	Slovakia	NA	NA	NA	NA	NA	NA
1027	Switzerland	NA	NA	NA	NA	NA	NA
1028	France	NA	NA	NA	NA	NA	NA

1029	Hungary	19	48	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1030	Hungary	19	48	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1031	UK	0	52	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1032	UK	0	52	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1033	France	NA	NA	NA	NA	NA	NA
1034	Czech republic	16.301	49.1	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1035	Czech republic	16.3332	49.1526	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1036	Austria	16.3845	48.5655	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1037	Romania	NA	NA	NA	NA	NA	NA
1038	Poland	NA	NA	NA	Cold	Without dry season	Warm summer
1039	Romania	NA	NA	NA	NA	NA	NA
1040	Romania	NA	NA	NA	NA	NA	NA
1041	Romania	NA	NA	NA	NA	NA	NA
1042	Romania	NA	NA	NA	NA	NA	NA
1043	Romania	27.05	47.8166667	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1044	Hungary	19	48	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1045	Hungary	19	48	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1046	Romania	NA	NA	NA	NA	NA	NA
1047	Canada	NA	NA	NA	NA	NA	NA
1048	USA	NA	NA	NA	NA	NA	NA
1049	Russia	NA	NA	NA	NA	NA	NA
1050	USA	NA	NA	NA	NA	NA	NA
1051	USA	NA	NA	NA	NA	NA	NA
1052	Romania	NA	NA	NA	NA	NA	NA
1053	UK	0	52	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1054	Romania	NA	NA	NA	NA	NA	NA
1055	Hungary	19	48	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1056	Hungary	19	48	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1057	USA	NA	NA	NA	NA	NA	NA
1058	Australia	NA	NA	NA	NA	NA	NA
1059	Iran	NA	NA	NA	NA	NA	NA
1060	Czech republic	NA	NA	Temperate Broadleaf Mixed Forests	NA	NA	NA
1061	Hungary	19	48	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1062	Hungary	19	48	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1063	Italy	NA	NA	NA	NA	NA	NA
1064	Italy	NA	NA	NA	NA	NA	NA

1065	Italy	NA	NA	NA	NA	NA	NA
1066	Italy	NA	NA	NA	NA	NA	NA
1067	Romania	NA	NA	NA	NA	NA	NA
1068	Romania	NA	NA	NA	NA	NA	NA
1069	Spain	NA	NA	NA	NA	NA	NA
1070	Syria	NA	NA	NA	NA	NA	NA
1071	UK	-1.25	51	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1072	UK	-1	51	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1073	UK	2	52	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1074	UK	-4.0666667	52.4166667	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1075	UK	-4.0666667	52.4166667	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1076	UK	-4.0666667	52.4166667	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1077	NA	NA	NA	NA	NA	NA	NA
1078	UK	-4.0666667	52.4166667	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1079	NA	NA	NA	NA	NA	NA	NA
1080	Romania	26.1	44.4333333	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1081	NA	NA	NA	NA	NA	NA	NA
1082	NA	NA	NA	NA	NA	NA	NA
1083	NA	NA	NA	NA	NA	NA	NA
1084	NA	NA	NA	NA	NA	NA	NA
1085	Poland	18	53.15	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1086	Poland	23.35	52.216667	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1087	NA	NA	NA	NA	NA	NA	NA
1088	Poland	23.35	52.216667	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1089	NA	NA	NA	NA	NA	NA	NA
1090	NA	NA	NA	NA	NA	NA	NA
1091	NA	NA	NA	NA	NA	NA	NA
1092	Poland	20.15	50.2	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1093	NA	NA	NA	NA	NA	NA	NA
1094	NA	NA	NA	NA	NA	NA	NA
1095	Former Soviet Union	NA	NA	NA	NA	NA	NA
1096	NA	NA	NA	NA	NA	NA	NA
1097	NA	NA	NA	NA	NA	NA	NA
1098	Spain	-0.666667	40.666667	Mediterranean	Arid	Steppe	Cold

				Forests, Woodlands Scrub			
1099	NA	NA	NA	NA	NA	NA	NA
1100	Armenia	45	40	Temperate Grasslands, Savannas Shrublands	Cold	Without dry season	Hot summer
1101	Armenia	45	40	Temperate Grasslands, Savannas Shrublands	Cold	Without dry season	Hot summer
1102	Armenia	45	40	Temperate Grasslands, Savannas Shrublands	Cold	Without dry season	Hot summer
1103	Turkey	NA	NA	NA	NA	NA	NA
1104	Turkey	27.23333	38.45	Mediterranean Forests, Woodlands Scrub	Temperate	Dry summer	Hot summer
1105	Turkey	30.28333	37.71667	Mediterranean Forests, Woodlands Scrub	Temperate	Dry summer	Hot summer
1106	Turkey	27.88333	39.65	Mediterranean Forests, Woodlands Scrub	Temperate	Dry summer	Hot summer
1107	Turkey	40.25	40.26667	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1108	Turkey	43.08333	40.6	Temperate Grasslands, Savannas Shrublands	Cold	Without dry season	Warm summer
1109	Turkey	30.53333	39.76667	Mediterranean Forests, Woodlands Scrub	Temperate	Dry summer	Warm summer
1110	Turkey	40.25	40.26667	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1111	Spain	NA	NA	NA	NA	NA	NA
1112	Spain	0.38	41.37	Mediterranean Forests, Woodlands Scrub	Arid	Steppe	Cold
1113	Spain	0.38	41.37	Mediterranean Forests, Woodlands Scrub	Arid	Steppe	Cold
1114	Spain	0.38	41.37	Mediterranean Forests, Woodlands Scrub	Arid	Steppe	Cold
1115	Turkey	32.51667	37.86667	Temperate Broadleaf Mixed Forests	Arid	Steppe	Cold
1116	UK	0	52	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1117	UK	0	52	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1118	Iran	NA	NA	NA	NA	NA	NA
1119	Turkey	35.86667	38.85	Temperate Broadleaf Mixed Forests	Cold	Dry summer	Warm summer
1120	Turkey	41.28333	39.91667	Temperate Grasslands, Savannas Shrublands	Cold	Without dry season	Warm summer

1121	Turkey	30.53333	39.76667	Mediterranean Forests, Woodlands Scrub	Temperate	Dry summer	Warm summer
1122	Turkey	30.7	36.88333	Mediterranean Forests, Woodlands Scrub	Temperate	Dry summer	Hot summer
1123	Turkey	30.7	36.88333	Mediterranean Forests, Woodlands Scrub	Temperate	Dry summer	Hot summer
1124	Turkey	32.86667	39.93333	Temperate Broadleaf Mixed Forests	Arid	Steppe	Cold
1125	Turkey	30.53333	39.76667	Mediterranean Forests, Woodlands Scrub	Temperate	Dry summer	Warm summer
1126	Greece	NA	NA	NA	NA	NA	NA
1127	USA	-117.16667	46.73333	Temperate Grasslands, Savannas Shrublands	Cold	Dry summer	Warm summer
1128	Iran	48.3013889	38.2494444	Temperate Conifer Forests	Temperate	Dry summer	Hot summer
1129	Turkey	NA	NA	NA	NA	NA	NA
1130	Iran	NA	NA	NA	NA	NA	NA
1131	Iran	50.6272222	32.77	Temperate Broadleaf Mixed Forests	Arid	Desert	Cold
1132	Former soviet union	NA	NA	NA	NA	NA	NA
1133	Iran	50.4166667	33.4166667	Temperate Broadleaf Mixed Forests	Arid	Steppe	Cold
1134	Iran	47	34.5	Temperate Broadleaf Mixed Forests	Arid	Steppe	Hot
1135	Iran	50	32	Temperate Broadleaf Mixed Forests	Arid	Desert	Hot
1136	Iran	47	35.3	Temperate Broadleaf Mixed Forests	Temperate	Dry summer	Hot summer
1137	Iran	48.3013889	38.2494444	Temperate Conifer Forests	Temperate	Dry summer	Hot summer
1138	Spain	-0.8	40.833333	Mediterranean Forests, Woodlands Scrub	Arid	Steppe	Cold
1139	Switzerland	6.3166667	46.6666667	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1140	Switzerland	7.1833333	46	Temperate Conifer Forests	Polar	Frost	
1141	Turkey	NA	NA	NA	NA	NA	NA
1142	Turkey	31.7166667	41.2166667	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1143	Iran	NA	NA	NA	NA	NA	NA
1144	Iran	NA	NA	NA	NA	NA	NA
1145	Iran	NA	NA	NA	NA	NA	NA
1146	Iran	NA	NA	NA	NA	NA	NA
1147	Iran	NA	NA	NA	NA	NA	NA
1148	Iran	48.3591667	32.46	Temperate Broadleaf Mixed Forests	Arid	Desert	Hot
1149	Serbia	NA	NA	NA	NA	NA	NA
1150	Italy	NA	NA	NA	NA	NA	NA

1151	Former soviet union	NA	NA	NA	NA	NA	NA
1152	Former soviet union	NA	NA	NA	NA	NA	NA
1153	Former soviet union	NA	NA	NA	NA	NA	NA
1154	Former soviet union	NA	NA	NA	NA	NA	NA
1155	Former soviet union	NA	NA	NA	NA	NA	NA
1156	Former soviet union	NA	NA	NA	NA	NA	NA
1157	Former soviet union	NA	NA	NA	NA	NA	NA
1158	Russia	42	46	Temperate Grasslands, Savannas Shrublands	Cold	Without dry season	Hot summer
1159	Former soviet union	NA	NA	NA	NA	NA	NA
1160	Former soviet union	NA	NA	NA	NA	NA	NA
1161	Former soviet union	NA	NA	NA	NA	NA	NA
1162	Former soviet union	NA	NA	NA	NA	NA	NA
1163	UK	0	52	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1164	UK	0	52	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1165	UK	0	52	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1166	UK	0	52	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1167	Former soviet union	NA	NA	NA	NA	NA	NA
1168	Former soviet union	NA	NA	NA	NA	NA	NA
1169	Lithuania	NA	NA	NA	Cold	Without dry season	Warm summer
1170	Ukraine	NA	NA	NA	NA	NA	NA
1171	Russia	49.4125	53.5233333	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1172	Russia	43.5	43.5	NA	Cold	Without dry season	Hot summer
1173	Ukraine	NA	NA	NA	NA	NA	NA
1174	Ukraine	NA	NA	NA	NA	NA	NA
1175	Russia	38.9769444	45.0327778	Temperate Grasslands, Savannas Shrublands	Cold	Without dry season	Hot summer
1176	Ukraine	NA	NA	NA	NA	NA	NA
1177	Spain	NA	NA	NA	NA	NA	NA
1178	Spain	NA	NA	NA	NA	NA	NA
1179	Spain	NA	NA	NA	NA	NA	NA
1180	Spain	NA	NA	NA	NA	NA	NA
1181	Italy	11	44.75	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Hot summer
1182	Spain	NA	NA	NA	NA	NA	NA
1183	Spain	NA	NA	NA	NA	NA	NA
1184	Spain	NA	NA	NA	NA	NA	NA
1185	Spain	NA	NA	NA	NA	NA	NA
1186	Spain	NA	NA	NA	NA	NA	NA
1187	Spain	NA	NA	NA	NA	NA	NA

1188	Germany	NA	NA	NA	NA	NA	NA
1189	Former soviet union	NA	NA	NA	NA	NA	NA
1190	Poland	NA	NA	NA	Cold	Without dry season	Warm summer
1191	Former soviet union	NA	NA	NA	NA	NA	NA
1192	Switzerland	NA	NA	NA	NA	NA	NA
1193	Russia	49.4125	53.5233333	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1194	Former soviet union	NA	NA	NA	NA	NA	NA
1195	Russia	38.9769444	45.0327778	Temperate Grasslands, Savannas Shrublands	Cold	Without dry season	Hot summer
1196	Russia	38.9769444	45.0327778	Temperate Grasslands, Savannas Shrublands	Cold	Without dry season	Hot summer
1197	Norway	NA	NA	NA	NA	NA	NA
1198	Former soviet union	NA	NA	NA	NA	NA	NA
1199	Former soviet union	NA	NA	NA	NA	NA	NA
1200	Germany	NA	NA	NA	NA	NA	NA
1201	Former soviet union	NA	NA	NA	NA	NA	NA
1202	Former soviet union	NA	NA	NA	NA	NA	NA
1203	Former soviet union	NA	NA	NA	NA	NA	NA
1204	Former soviet union	NA	NA	NA	NA	NA	NA
1205	Italy	NA	NA	NA	NA	NA	NA
1206	Former soviet union	NA	NA	NA	NA	NA	NA
1207	Bulgaria	NA	NA	Temperate Broadleaf Mixed Forests	NA	NA	NA
1208	Former soviet union	NA	NA	NA	NA	NA	NA
1209	Former soviet union	NA	NA	NA	NA	NA	NA
1210	Switzerland	NA	NA	NA	NA	NA	NA
1211	Switzerland	NA	NA	NA	NA	NA	NA
1212	Switzerland	NA	NA	NA	NA	NA	NA
1213	Switzerland	NA	NA	NA	NA	NA	NA
1214	Switzerland	NA	NA	NA	NA	NA	NA
1215	Spain	-3.65	42.766667	Mediterranean Forests, Woodlands Scrub	Temperate	Without dry season	Warm summer
1216	Spain	-3.866667	42.75	Mediterranean Forests, Woodlands Scrub	Temperate	Without dry season	Warm summer
1217	Spain	-3.7	42.7	Mediterranean Forests, Woodlands Scrub	Temperate	Without dry season	Warm summer
1218	Spain	NA	NA	NA	NA	NA	NA
1219	Romania	28.6166667	44.9833333	Temperate Grasslands, Savannas Shrublands	Cold	Without dry season	Hot summer
1220	Morocco	NA	NA	NA	NA	NA	NA
1221	Turkey	32.8333333	39.9166667	Temperate Broadleaf Mixed	Arid	Steppe	Cold

				Forests			
1222	Turkey	32.8333333	39.9166667	Temperate Broadleaf Mixed Forests	Arid	Steppe	Cold
1223	Turkey	32.8333333	39.9166667	Temperate Broadleaf Mixed Forests	Arid	Steppe	Cold
1224	Czech Republic	15	50	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1225	Czech Republic	15	50	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1226	Czech Republic	15	50	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1227	Czech Republic	15	50	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1228	Czech Republic	15	50	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1229	Czech Republic	15	50	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1230	Czech Republic	15	50	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1231	Czech Republic	15	50	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1232	Iran	48	33.5	Temperate Broadleaf Mixed Forests	Arid	Desert	Hot
1233	Iran	48.25	36.5833333	Temperate Broadleaf Mixed Forests	Temperate	Dry summer	Hot summer
1234	Turkey	NA	NA	NA	NA	NA	NA
1235	Turkey	NA	NA	NA	NA	NA	NA
1236	Turkey	NA	NA	NA	NA	NA	NA
1237	Turkey	39.26	40.26	Temperate Conifer Forests	Cold	Without dry season	Warm summer
1238	Turkey	37.01	39.44	Temperate Broadleaf Mixed Forests	Cold	Dry summer	Warm summer
1239	Zimbabwe	32.583333	-18.383333	Montane Grasslands Shrublands	Temperate	Dry winter	Hot summer
1240	Zimbabwe	NA	NA	NA	NA	NA	NA
1241	USA	NA	NA	NA	NA	NA	NA
1242	Romania	NA	NA	NA	NA	NA	NA
1243	Romania	NA	NA	NA	NA	NA	NA
1244	Russia	39.7138889	47.2363889	Temperate Grasslands, Savannas Shrublands	Cold	Without dry season	Hot summer
1245	Kazakhstan	71.427778	51.181111	Temperate Grasslands, Savannas Shrublands	Cold	Without dry season	Warm summer
1246	Kazakhstan	70.999444	51.700556	Temperate Grasslands, Savannas Shrublands	Cold	Without dry season	Warm summer
1247	Kazakhstan	71.427778	51.181111	Temperate Grasslands, Savannas Shrublands	Cold	Without dry season	Warm summer
1248	Romania	NA	NA	NA	NA	NA	NA
1249	Romania	NA	NA	NA	NA	NA	NA
1250	Romania	NA	NA	NA	NA	NA	NA

1251	Romania	NA	NA	NA	NA	NA	NA
1252	Russia	NA	NA	NA	NA	NA	NA
1253	Turkey	43.96667	37.7	Temperate Grasslands, Savannas Shrublands	Cold	Dry summer	Hot summer
1254	Turkey	43.85	38.28333	Temperate Grasslands, Savannas Shrublands	Cold	Dry summer	Hot summer
1255	Turkey	35.9333333	36.1166667	Mediterranean Forests, Woodlands Scrub	Temperate	Dry summer	Hot summer
1256	Turkey	30.6666667	38.75	Mediterranean Forests, Woodlands Scrub	Temperate	Dry summer	Warm summer
1257	Turkey	36.5833333	40.4166667	Temperate Conifer Forests	Temperate	Dry summer	Hot summer
1258	China	NA	NA	NA	NA	NA	NA
1259	China	85.456111	42.569167	Montane Grasslands Shrublands	Arid	Steppe	Cold
1260	China	87.85	43.8	Montane Grasslands Shrublands	Arid	Steppe	Cold
1261	Armenia	NA	NA	NA	NA	NA	NA
1262	UK	-2	52	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1263	Armenia	NA	NA	NA	NA	NA	NA
1264	Armenia	NA	NA	NA	NA	NA	NA
1265	Armenia	NA	NA	NA	NA	NA	NA
1266	Poland	NA	NA	NA	Cold	Without dry season	Warm summer
1267	UK	0	52	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1268	UK	0	52	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1269	UK	0	52	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Warm summer
1270	Italy	NA	NA	NA	NA	NA	NA
1271	Italy	NA	NA	NA	NA	NA	NA
1272	France	NA	NA	NA	NA	NA	NA
1273	NA	NA	NA	NA	NA	NA	NA
1274	Italy	NA	NA	NA	NA	NA	NA
1275	Czech Republic	NA	NA	NA	NA	NA	NA
1276	Bulgaria	NA	NA	Temperate Broadleaf Mixed Forests	NA	NA	NA
1277	Turkey	NA	NA	Temperate Broadleaf Mixed Forests	NA	NA	NA
1278	NA	NA	NA	NA	NA	NA	NA
1279	Spain	NA	NA	NA	NA	NA	NA
1280	Spain	NA	NA	NA	NA	NA	NA
1281	Spain	NA	NA	NA	NA	NA	NA
1282	Spain	NA	NA	NA	NA	NA	NA
1283	Spain	NA	NA	NA	NA	NA	NA
1284	Spain	NA	NA	NA	NA	NA	NA
1285	Spain	NA	NA	NA	NA	NA	NA
1286	Spain	NA	NA	NA	NA	NA	NA
1287	Spain	NA	NA	NA	NA	NA	NA

1288	Spain	NA	NA	NA	NA	NA	NA
1289	France	6	45	Temperate Conifer Forests	Cold	Without dry season	Cold summer
1290	France	NA	NA	NA	NA	NA	NA
1291	France	NA	NA	NA	NA	NA	NA
1292	Poland	NA	NA	NA	Cold	Without dry season	Warm summer
1293	NA	NA	NA	NA	NA	NA	NA
1294	NA	NA	NA	NA	NA	NA	NA
1295	NA	NA	NA	NA	NA	NA	NA
1296	NA	NA	NA	NA	NA	NA	NA
1297	NA	NA	NA	NA	NA	NA	NA
1298	Armenia	NA	NA	NA	NA	NA	NA
1299	Armenia	NA	NA	NA	NA	NA	NA
1300	Armenia	NA	NA	NA	NA	NA	NA
1301	Armenia	NA	NA	NA	NA	NA	NA
1302	Armenia	NA	NA	NA	NA	NA	NA
1303	Armenia	NA	NA	NA	NA	NA	NA
1304	NA	NA	NA	NA	NA	NA	NA
1305	Georgia	NA	NA	NA	NA	NA	NA
1306	France	NA	NA	NA	NA	NA	NA
1307	Russia	NA	NA	NA	NA	NA	NA
1308	Germany	NA	NA	NA	NA	NA	NA
1309	Iran	NA	NA	NA	NA	NA	NA
1310	France	NA	NA	NA	NA	NA	NA
1311	Turkmenistan	NA	NA	NA	NA	NA	NA
1312	Kazakhstan	NA	NA	NA	NA	NA	NA
1313	Georgia	NA	NA	NA	NA	NA	NA
1314	Former soviet union	NA	NA	NA	NA	NA	NA
1315	Russia	NA	NA	NA	NA	NA	NA
1316	France	NA	NA	NA	NA	NA	NA
1317	NA	NA	NA	NA	NA	NA	NA
1318	Former soviet union	NA	NA	NA	NA	NA	NA
1319	NA	NA	NA	NA	NA	NA	NA
1320	Armenia	NA	NA	NA	NA	NA	NA
1321	Former soviet union	NA	NA	NA	NA	NA	NA
1322	Armenia	NA	NA	NA	NA	NA	NA
1323	Armenia	NA	NA	NA	NA	NA	NA
1324	Armenia	NA	NA	NA	NA	NA	NA
1325	Armenia	NA	NA	NA	NA	NA	NA
1326	Armenia	NA	NA	NA	NA	NA	NA
1327	Armenia	NA	NA	NA	NA	NA	NA
1328	Former soviet union	69.13	41.16	Temperate Grasslands, Savannas Shrublands	Cold	Dry summer	Hot summer
1329	Former soviet union	NA	NA	NA	NA	NA	NA
1330	Bulgaria	24.83833	41.99361	Temperate Broadleaf Mixed Forests	Polar	Tundra	
1331	Bulgaria	24.5666667	41.65	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Cold summer
1332	Bulgaria	24.80028	41.99528	Temperate Broadleaf Mixed	Polar	Tundra	

				Forests			
1333	Former soviet union	NA	NA	NA	NA	NA	NA
1334	Former soviet union	NA	NA	NA	NA	NA	NA
1335	Former soviet union	NA	NA	NA	NA	NA	NA
1336	Russia	NA	NA	NA	NA	NA	NA
1337	Russia	83.05	55.04	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1338	Mongolia	105.53889	49.86306	Temperate Grasslands, Savannas Shrublands	Cold	Dry Winter	Cold summer
1339	Mongolia	105.29	49.71806	Temperate Grasslands, Savannas Shrublands	Cold	Dry Winter	Cold summer
1340	Iran	51.8333333	32.8333333	Temperate Broadleaf Mixed Forests	Arid	Desert	Cold
1341	Hungary	19	48	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1342	Former soviet union	NA	NA	NA	NA	NA	NA
1343	Former soviet union	NA	NA	NA	NA	NA	NA
1344	Former soviet union	34.09	44.5	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Hot summer
1345	Russia	43.01056	43.46028	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Hot summer
1346	Former soviet union	44.48	41.43	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Hot summer
1347	Former soviet union	44.48	41.43	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Hot summer
1348	Former soviet union	44.48	41.43	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Hot summer
1349	Russia	40.82333	44.15889	Temperate Broadleaf Mixed Forests	Temperate	Without dry season	Hot summer
1350	Russia	36.96583	45.27861	Temperate Grasslands, Savannas Shrublands	Temperate	Without dry season	Hot summer
1351	Bulgaria	24.26361	42.48333	Temperate Broadleaf Mixed Forests	Cold	Without dry season	Warm summer
1352	Pakistan	70.49	32.2	Deserts Xeric Shrublands	Arid	Desert	Hot
1353	Former soviet union	NA	NA	NA	NA	NA	NA
1354	Former soviet union	NA	NA	NA	NA	NA	NA
1355	Former soviet union	NA	NA	NA	NA	NA	NA
1356	Spain	NA	NA	NA	NA	NA	NA
1357	Spain	NA	NA	NA	NA	NA	NA
1358	Armenia	NA	NA	NA	NA	NA	NA
1359	Armenia	NA	NA	NA	NA	NA	NA
1360	Armenia	NA	NA	NA	NA	NA	NA

APPENDIX 3: PREDICTED MEANS USING SPATIAL ANALYSIS OF REML FOR AGRONOMIC EVALUATIONS

Accession	Fusarium_oct2008	Fusarium_jul2009	Mildew_oct2008
1001	1.931	0.967	0.34356
1003	4.954	1.0597	0.00939
1004	2.715	1.0536	0.00565
1005	2.441	1.0581	0.3499
1007	5.13	0.544	-0.00628
1008	2.946	0.9434	0.01499
1009	1.555	1.2233	0.00083
1012	3.067	0.6944	-0.01838
1013	1.715	1.0323	0.33155
1016	0.457	0.5332	0.02112
1017	1.352	1.0245	-0.01093
1018	0.903	0.9333	-0.01542
1019	1.956	0.968	0.00976
1020	0.925	0.9115	0.00131
1024	1.231	0.5467	0.01774
1026	1.844	0.9366	-0.02847
1028	-0.341	0.9092	0.02002
1035	1.705	0.6335	-0.00773
1036	0.608	1.0627	-0.04378
1040	1.808	1.0622	-0.01627
1041	1.433	0.6863	-0.00987
1042	2.042	0.9458	0.01752
1043	0.994	0.9306	-0.00225
1044	1.963	0.5769	0.00243
1045	3.023	0.554	-0.00058
1046	2.794	1.0967	0.00347
1071	2.432	0.696	0.31119
1072	0.595	1.0582	0.49112
1077	2.851	0.9763	0.02929
1095	3.73	0.9199	0.04106
1100	1.498	1.0065	-0.01228
1101	0.672	0.9849	-0.00606
1102	10.284	0.9994	-0.01692
1103	4.464	0.9924	-0.02431
1104	1.787	1.0291	0.00137
1105	6.706	1.0153	0.01743
1106	5.075	0.9977	-0.00024
1107	6.05	0.9984	0.01391
1108	5.609	0.514	0.00004
1110	1.449	0.9828	-0.00404
1111	4.98	1.0677	-0.00182
1112	5.158	0.99	0.00853
1113	3.219	0.9789	-0.00951

1114	8.463	1.0188	-0.01344
1115	2.631	0.9817	0.00386
1116	5.302	1.0004	0.00715
1117	3.758	1.0457	0.00389
1118	2.237	0.6493	0.01532
1119	3.469	1.0537	0.00624
1120	10.081	1.0247	-0.00333
1121	3.362	0.6358	-0.02994
1122	3.981	1.0286	0.00007
1123	6.222	0.9945	-0.00386
1124	7.085	1.014	0.02711
1125	2.107	0.9848	0.50132
1127	1.174	1.0114	-0.00177
1128	3.698	1.0073	0.03328
1129	2.151	0.9948	0.01056
1131	2.531	0.9751	-0.0172
1132	0.718	1.012	0.49753
1133	4.396	1.0142	-0.01214
1134	5.648	1.0179	0.01998
1136	3.046	0.9475	0.00625
1137	2.125	0.9559	0.00114
1138	1.388	0.9415	-0.00584
1139	2.117	0.9552	1.01415
1140	2.742	0.9352	0.02846
1141	4.932	0.9741	-0.00095
1142	6.866	0.9785	-0.02234
1143	2.281	0.9651	-0.00235
1145	1.692	1.0194	0.00513
1148	1	0.9483	0.00831
1151	2.126	0.9747	0.00057
1152	1.669	0.9797	-0.01421
1153	5.204	0.9806	0.00461
1154	1.296	0.9621	0.00382
1155	6.585	0.9789	0.00423
1156	2.158	0.9515	0.01583
1157	2.191	0.9563	0.00617
1158	3.609	0.9567	0.00555
1159	4.603	0.9568	0.0001
1160	6.861	0.9602	0.02
1161	0.778	0.9358	-0.01063
1163	0.779	0.9861	-0.01975
1164	4.818	0.9662	-0.00322
1165	4.576	0.6353	0.65269
1166	4.113	0.9533	0.00608
1167	2.976	0.957	0.04144
1168	1.216	0.9558	0.00469
1169	3.614	0.9581	-0.00918
1170	5.146	0.9388	-0.0016
1171	2.247	0.9498	-0.00928
1172	1.338	1.0341	0.02332
1173	0.362	1.0514	0.5096
1174	0.686	0.9611	-0.00294

1175	0.542	0.9439	-0.00474
1176	7.209	1.0007	-0.00549
1177	-0.04	-0.0576	0.00063
1179	-0.093	-0.0554	0.02943
1180	6.735	0.9811	-0.00886
1181	0.926	0.4913	0.00093
1183	3.976	0.9956	0.01543
1184	-0.283	-0.0537	-0.01234
1185	-0.364	-0.0637	-0.02136
1187	2.039	0.9926	0.00321
1188	1.874	0.9785	0.00454
1189	2.707	0.9795	0.01496
1190	0.931	0.6485	0.00068
1191	0.612	0.9483	0.00488
1193	3.257	0.975	-0.00877
1194	2.45	0.9809	0.00758
1195	1.774	0.9818	0.00109
1196	1.19	1.0167	-0.00002
1197	2.547	0.9871	0.00052
1198	1.569	0.9754	-0.01001
1199	3.929	0.9836	0.00386
1200	7.396	0.4666	-0.00371
1201	4.065	0.9887	-0.0082
1202	1.874	0.9837	0.01025
1203	-1.382	0.9859	0.99824
1204	1.799	0.9906	0.00411
1205	1.614	0.9833	0.00053
1206	1.438	0.4669	0.98748
1207	1.778	0.9645	1.02122
1209	3.037	0.9622	-0.00632
1210	2.186	0.9885	0.47079
1211	7.795	0.9724	-0.04456
1212	4.454	0.9587	-0.01676
1213	1.945	0.9638	-0.00755
1214	4.599	0.9586	0.00697
1218	3.005	0.9841	-0.00958
1219	3.264	0.9808	-0.03283
1220	2.624	0.9702	-0.01229
1221	1.293	0.9359	0.00507
1228	0.791	0.9608	-0.00049
1230	0.765	0.9736	0.00081
1231	1.157	0.4211	-0.00033
1233	1.784	0.9976	-0.00337
1241	4.641	0.9917	-0.0234
1245	3.307	0.9531	0.00966
1247	0.616	0.4565	-0.00697
1248	1.583	0.9935	0.00113
1249	0.277	0.4784	-0.00471
1250	0.278	0.9712	0.50921
1252	2.798	0.9843	-0.01019
1253	3.545	0.9815	0.01443
1254	0.33	1.0528	-0.0309

1256	-0.146	-0.0589	0.50631
1258	4.978	0.907	-0.01634
1259	1.375	0.9742	-0.01612
1260	2.978	0.9736	-0.00914
1261	2.326	0.9769	0.01917
1262	1.941	1.0483	0.67552
1263	1.661	0.7019	0.00789
1264	1.891	1.0175	0.00922
1265	2.608	0.4385	0.03449
1266	1.164	1.0987	-0.04287
1289	0.54	0.545	-0.01023
1290	0.493	1.0721	0.02141
1291	0.465	0.5552	0.00509
1292	0.122	0.5129	0.01476
1300	NA	0.5151	0.00515

Accession	Floweringdate 2008	Floweringdate 2009	Flowerpresence oct2008	Flowerpresence jul2009
1001	136.7	137.4	0.0417	1.394
1003	153.3	140.8	0.6178	1.983
1004	153.4	147	1.1026	2.043
1005	149.5	141	0.6889	1.988
1007	144	144.5	0.6027	1.998
1008	148	138.7	0.0875	2.08
1009	154.8	146.4	1.1829	1.947
1012	149.8	146.7	0.433	2.048
1013	148.5	138.3	0.1354	2.034
1016	162.4	144.4	0.504	2.039
1017	146.6	135.3	0.1419	2.071
1018	150.3	139.5	1.0517	2.038
1019	150.9	141.1	0.4128	2.059
1020	143.8	141.8	-0.169	2.036
1024	141.2	139	0.6543	2.029
1026	NA	145.8	0.3499	2.045
1028	150.1	134.9	0.8273	2.019
1035	NA	146	0.5803	2.056
1036	163.2	152.8	0.6276	1.676
1040	151	144.3	0.144	2.032
1041	151.5	145.1	0.1944	2.042
1042	150.2	146.7	0.4727	2.039
1043	154.6	147.6	-0.0814	2.012
1044	149.6	142	0.1385	2.046
1045	154.9	146.1	0.1501	2.008
1046	151.2	146.8	0.4447	1.96
1071	134.6	140.6	0.4536	0.803
1072	157.1	151.2	0.0846	1.993
1077	162.2	148.4	-0.0891	1.354
1095	151.9	141.6	-0.0516	2.058
1100	147.6	145.1	0.9302	2.04
1101	149.8	149	-0.0123	2.058
1102	144.6	144.6	0.9328	1.951
1103	153.4	144.8	-0.062	1.987

1104	153.6	146.9	0.632	1.778
1105	146.2	147.9	-0.1856	1.603
1106	146.4	146.2	-0.1132	1.677
1107	152.4	146.3	-0.0905	1.93
1108	152.7	144	0.8448	1.998
1110	151.9	146.6	0.0465	2
1111	136.2	138.8	0.9349	2.019
1112	147.7	140.7	0.9245	1.655
1113	149.7	138.5	0.6569	2.01
1114	153.7	145.1	-0.0471	1.963
1115	151.9	146	0.3177	2.01
1116	137.7	139	0.3198	2.034
1117	135.1	138.2	0.4742	2.035
1118	151.3	145.9	0.9501	1.996
1119	151.8	147.1	-0.1159	2.055
1120	153.2	146.1	-0.0396	1.97
1121	149.6	142.4	0.6455	1.682
1122	143.7	137.9	0.3783	2.018
1123	150.8	145.6	0.3462	2.037
1124	148.7	142.6	0.4495	1.998
1125	143.9	139.1	0.5623	2.037
1127	144.6	142.7	-0.05	1.954
1128	153.1	146.3	-0.0728	1.988
1129	159	145.6	0.5623	2.023
1131	152	144.7	-0.1001	2
1132	136	139.3	-0.0544	1.995
1133	151.2	142	0.425	1.982
1134	146.5	146.8	0.4235	2.018
1136	153.1	143	0.5768	1.937
1137	152.9	142	0.4821	1.985
1138	139.4	135.2	0.5262	1.929
1139	133.5	136.1	0.5047	1.94
1140	136.8	133.2	0.1175	0.995
1141	143.1	143.2	0.9681	1.985
1142	149	141.1	0.9591	1.958
1143	152.1	146.7	0.891	1.934
1145	148.6	144	0.9211	1.968
1148	145.1	143.1	1.1678	1.993
1151	150.6	145.8	0.5236	1.97
1152	151.7	147.9	0.036	1.536
1153	153.1	142.9	0.543	1.995
1154	152.2	145.5	0.0349	1.956
1155	152.7	147.3	0.4551	1.978
1156	150.5	143.5	0.5905	1.639
1157	151.5	145.4	0.077	1.947
1158	152.3	146.1	0.6693	1.959
1159	153.6	147.9	0.5304	1.936
1160	150.9	143.4	0.5114	1.455
1161	152.2	144.6	0.1076	2.024
1163	150.8	142.7	1.0033	1.97
1164	150.5	141.3	-0.0551	1.973
1165	138.7	135.7	-0.0105	1.024

1166	154.2	144.5	0.4585	1.934
1167	149	144.1	0.0176	1.936
1168	150.2	141.6	0.0495	2.018
1169	138.3	139	0.0025	1.943
1170	142.3	141.6	-0.0224	1.341
1171	150.2	143	-0.0366	1.929
1172	152.9	146.8	0.4367	2.025
1173	150.7	141.7	-0.0146	1.573
1174	153.7	143.2	-0.1199	2.074
1175	NA	149.3	-0.1106	0.997
1176	151.8	142.9	0.0675	1.949
1177	150.4	145.2	0.4611	1.001
1179	NA	152.3	0.3773	1.687
1180	148.7	141.4	-0.0226	1.988
1181	151.4	141.7	0.4633	2.003
1183	147.4	138.5	0.014	2.035
1184	NA	154.4	0.0301	1.924
1185	NA	154.3	-0.1434	1.536
1187	136.3	138.4	0.0235	1.649
1188	141.4	141.2	-0.0592	1.615
1189	149.5	144.8	-0.0607	1.303
1190	141.6	141.7	0.3954	2.024
1191	150.9	139.9	0.0931	2.004
1193	159.4	148	-0.0328	1.937
1194	147.9	140.2	0.5281	1.916
1195	150.2	141.4	0.5047	1.947
1196	146.8	141.4	-0.0074	1.62
1197	152.5	144	0.0115	1.601
1198	147	141.1	-0.0082	1.994
1199	156	143.2	0.0544	1.993
1200	142.8	138.9	0.5445	1.969
1201	150.5	146.9	0.5139	1.591
1202	137.7	142	-0.0102	1.317
1203	142.2	141.5	1.0763	1.938
1204	146.7	140.6	-0.0123	1.669
1205	141.9	142.5	0.4333	1.941
1206	149.4	141.3	0.1305	1.498
1207	137.2	137.4	0.0061	1.578
1209	154.7	146.5	0.5231	1.621
1210	130.9	135.7	0.0065	1.311
1211	147.3	137.8	0.9589	1.981
1212	153.7	144.7	0.9888	1.945
1213	147.5	137.2	0.042	1.988
1214	148.9	141	0.4384	1.65
1218	147.2	141.3	-0.0223	1.937
1219	158.1	148.9	0.4561	1.408
1220	134.8	141.6	0.4951	1.975
1221	155.8	144.5	0.0122	1.672
1228	149.1	142	0.984	1.974
1230	NA	146.6	0.4699	1.993
1231	151.8	138.4	-0.1317	2.073
1233	152	143.3	0.9389	2.081

1241	157.4	145.9	-0.1251	2.015
1245	164.1	150.3	0.5754	1.948
1247	163.6	150.8	-0.1526	1.509
1248	162.7	147.6	-0.0673	1.535
1249	163.3	155.1	-0.1502	2.027
1250	147	143.4	0.3975	2.024
1252	162.7	150	-0.0334	2.015
1253	152.7	146.4	-0.0547	1.737
1254	151.1	142.4	0.4454	2.034
1256	165.7	166.4	0.378	0.717
1258	154.7	146.4	-0.155	2.034
1259	155.1	148.7	0.3338	2.036
1260	150.5	146.7	0.3066	2.051
1261	149.8	145.2	0.3723	2.053
1262	140.9	139.1	0.1954	1.038
1263	148.2	144	0.8339	2.01
1264	146.7	145.5	0.2319	1.562
1265	152.5	143.7	-0.152	1.516
1266	148.8	146.5	1.1354	2.047
1289	161.3	133.2	-0.0288	1.031
1290	146	140.5	0.4301	1.983
1291	150.1	135.4	0.4576	2.013
1292	165.5	142.5	-0.0676	2.016
1300	NA	147.3	0.3894	1.024

Accession	Dmweight 2008	DMweight 2009	Height 2008	Soilcover oct2008	Survival oct2008	Survival apr2009
1001	691.2	810.2	63.36	7.044	7.811	24.05
1003	853.4	NA	101.12	6.806	6.846	21.09
1004	1410.2	NA	102.36	5.715	6.058	16.86
1005	812.6	759	71.65	5.267	5.45	13.81
1007	1281	625.4	96.21	6.437	6.358	19.54
1008	825.4	NA	73.35	6.2	5.996	20.92
1009	1979.8	NA	100.58	3.344	6.882	28.39
1012	937.6	577.8	93.27	4.98	5.783	12.9
1013	1402.4	767.4	62.67	5.409	5.29	11.93
1016	354.2	NA	45.76	6.02	6.76	28.5
1017	1077.2	834.2	74.88	5.957	5.819	14.26
1018	1415.8	615.4	86.88	6.554	6.376	20.42
1019	1173.4	1513.8	92.9	7.95	7.731	29.37
1020	324.8	NA	55.26	4.566	5.501	24.59
1024	1085.6	NA	64.74	5.352	5.405	17.92
1026	1093	752	87.16	6.259	6.059	22.57
1028	709	667.6	69.48	5.903	6.425	11.59

1035	885.4	NA	81.46	6.409	6.829	20.67
1036	363.2	NA	47.74	5.075	6.594	24.05
1040	1970.6	NA	101.6	6.666	6.873	18.24
1041	1156	830.2	90.53	6.75	7.021	22.01
1042	1692.8	NA	99.31	5.972	6.478	14.56
1043	1228.8	667	95.04	6.145	7.476	22.64
1044	2028.4	NA	113.81	6.241	7.736	28.02
1045	1153	NA	96.14	6.214	7.495	23.14
1046	1327.2	NA	124.35	6.309	6.26	19.08
1071	1253.6	559.4	69.68	5.608	6.688	20.25
1072	749.6	NA	60.85	5.32	7.208	20.22
1077	1173.4	339.4	113.04	5.406	6.273	14.66
1095	981.6	NA	70.03	6.071	7.292	24.02
1100	1365.4	NA	98.29	5.85	6.755	16.52
1101	1212	NA	101.81	4.434	5.896	14.22
1102	475.4	NA	77.51	5.464	5.51	6.77
1103	651.8	580.6	92.83	6.061	7.016	21.24
1104	757.6	656.2	106.67	5.835	5.636	13.82
1105	738	NA	102.86	4.876	5.048	8.58
1106	616.4	NA	91.79	4.538	5.244	8.71
1107	1353.8	NA	87.34	5.756	6.855	20.08
1108	293.8	NA	84.54	5.241	5.94	9.47
1110	1696	420.4	90.73	4.441	5.577	19.1
1111	390.6	NA	72.58	5.964	4.398	13.35
1112	531.8	NA	84.14	4.584	4.194	13.72
1113	1471.2	478.4	91.74	6.657	6.259	16.58
1114	NA	NA	82.97	4.479	3.752	10.03
1115	1076	NA	108.63	7.244	6.883	21.25
1116	398.2	NA	62.73	5.039	4.333	10.85
1117	774.6	NA	66.04	5.799	5.126	10.28
1118	1579.2	441.4	85.69	5.503	6.633	15.36
1119	317	NA	90.15	5.196	4.684	9.58
1120	810.4	NA	98.36	5.481	6.434	15.36
1121	1196.8	NA	85.63	5.767	6.183	14.37
1122	790.8	NA	76.99	5.746	6.142	16.03
1123	797.8	419.8	68.28	5.078	5.336	15.54
1124	200.4	NA	75.17	4.023	5.255	10.89

1125	928	NA	77.81	5.663	7.073	19.18
1127	483.2	486.2	100.9	5.028	4.995	12.81
1128	1531.8	NA	82.94	4.818	4.643	12.08
1129	840.4	NA	82.12	2.995	2.112	8.59
1131	1200.6	NA	87.04	4.945	5.232	16.47
1132	850	NA	74.8	5.432	5.478	13.24
1133	1066.6	NA	98.75	5.473	6.032	18.35
1134	398	NA	95.23	3.912	3.91	9.16
1136	821.8	NA	77.04	5.994	6.394	17.62
1137	310.6	NA	83.07	4.765	4.496	16.71
1138	498	NA	69.52	6.063	6.195	17.96
1139	673	NA	59.41	6.181	6.242	19.35
1140	474	525.4	53.4	5.695	6.455	13.8
1141	211.2	NA	80.83	6.028	6.254	13.74
1142	830.4	NA	72.86	5.024	4.752	11.25
1143	725.4	NA	87.26	6.076	6.203	21.96
1145	518.4	NA	88.99	5.533	6.079	19.31
1148	925.6	NA	91.54	5.787	6.374	20.57
1151	1210	NA	104.65	5.545	5.532	14.1
1152	766.8	NA	98.91	5.203	5.897	11.65
1153	801.2	NA	82.64	6.627	6.952	13.42
1154	792.4	NA	95.91	5.999	5.792	16.32
1155	371.4	NA	108.47	6.722	6.396	15.37
1156	1324.4	961.6	97.82	5.388	5.896	15.67
1157	1007.6	358	104.61	4.084	5.159	12.36
1158	935.8	NA	101.11	5.283	5.063	17.35
1159	732.4	NA	94.31	4.953	5.97	18.86
1160	591	NA	92.18	5.338	5.276	15.08
1161	1356.6	NA	89.16	5.652	5.913	21.08
1163	1147.8	615.2	85.15	5.19	5.757	12.5
1164	715.8	NA	89.86	6.048	5.664	15.54
1165	386	736.2	59.72	7.114	7.041	22.42
1166	661.4	NA	98.33	5.296	5.794	13.99
1167	1114	NA	93.6	5.5	5.673	20.53
1168	1417.2	NA	89.31	4.999	7.288	23.83
1169	502.6	969.8	68.22	6.051	6.262	20.61
1170	1040.4	NA	85.31	6.176	5.866	19.46

1171	1427.4	895.6	92.32	5.235	6.31	13.83
1172	1004.4	NA	87.37	5.65	7.009	22.51
1173	1049.4	NA	94.6	6.279	7.033	19.92
1174	724.8	NA	82.4	6.43	7.035	17.63
1175	1267.8	NA	102.39	4.961	5.829	18.31
1176	548.2	NA	89.63	5.321	5.851	20.47
1177	684	NA	71.11	5.713	4.981	21.26
1179	707.8	92.4	34.9	4.708	6.694	20.9
1180	509.4	NA	71.67	5.84	6.126	20.89
1181	371.6	NA	93.8	6.094	6.758	22.46
1183	498.8	NA	74.07	5.21	5.742	17.97
1184	482.8	NA	54.66	2.814	3.215	14.39
1185	1166.6	NA	39.88	5.559	7.622	24.08
1187	839	NA	82.57	6.346	6.069	21.57
1188	535.6	NA	77.98	6.31	5.699	19.9
1189	1351.4	NA	92.66	6.086	4.938	11.26
1190	675	NA	86.98	6.105	5.756	15.54
1191	945.2	NA	93.39	5.925	5.599	18.26
1193	844	NA	89.33	5.957	6.73	22.16
1194	387.8	NA	79.45	5.66	5.997	21.26
1195	844.6	NA	99.69	7.081	6.176	20.76
1196	765.4	NA	79.76	6.263	6.274	24.97
1197	1042.2	1032.8	94.61	5.826	5.791	21.63
1198	31	NA	70.65	5.739	5.31	18.79
1199	1261.2	611.6	101.64	5.643	5.441	17.84
1200	1004.6	382	76.43	5.528	5.677	19.91
1201	1213.6	NA	108.79	6.778	6.345	22.17
1202	739.6	NA	91.45	6.41	5.813	15.76
1203	330.4	NA	71.64	6.394	5.224	27.22
1204	238.8	NA	61.68	6.91	6.953	16.94
1205	444.8	NA	86.48	6.058	5.917	12.37
1206	1203.6	NA	76.75	6.598	6.925	20.45
1207	465.4	NA	66.71	6.387	6.791	13.88
1209	1171.6	NA	109.15	5.643	6.577	10.3
1210	1041.4	632.2	64.09	7.278	6.481	18.51
1211	1200	605	83.83	5.804	5.6	16.28
1212	774.4	NA	87.71	4.826	6.151	14.07

1213	471.2	517.4	88.93	6.507	7.187	25.01
1214	570.6	NA	84.1	5.61	5.164	12.78
1218	300.2	NA	70.21	4.808	5.395	8.15
1219	812	NA	107.55	4.608	6.046	14.74
1220	873.8	512.6	83.27	4.576	4.478	14.23
1221	899.8	NA	86.96	4.53	4.639	17.44
1228	1121.6	NA	79.78	6.456	6.944	27.1
1230	972.4	658.2	85.91	7.995	7.921	22.85
1231	1001.8	NA	76.89	6.149	6.503	23.38
1233	143	NA	73.16	5.304	5.854	13.57
1241	734.4	340	91.61	6.083	6.319	15.29
1245	745.2	336.2	83.59	5.34	5.163	17.24
1247	1110.4	NA	81.39	5.076	5.261	16.03
1248	1035	NA	108.89	6.582	6.156	25.55
1249	1021.2	NA	100.16	4.829	6.434	22.14
1250	1096	NA	79.53	5.251	6.868	20.39
1252	1057.2	NA	104.86	5.67	6.239	18.66
1253	897.4	581	92.96	4.608	5.079	16.73
1254	862.4	NA	72.51	5.752	7.331	20.48
1256	880.2	217	76.45	2.613	6.899	24.78
1258	1018.2	NA	98.14	5.494	5.899	10.9
1259	605.2	405	93.35	5.312	5.142	14.42
1260	1352	642.4	106.56	5.33	5.338	14.37
1261	863	605.6	82.47	6.354	6.33	19.89
1262	1489	575.6	72.08	6.636	7.05	20.41
1263	1208.4	NA	89	6.127	6.45	20.62
1264	1439.6	716.8	92.71	6.097	6.768	18.94
1265	1028.4	NA	75.28	5.343	6.434	17.7
1266	2070.4	949.8	112.3	6.66	7.619	18.06
1289	218.4	NA	52.11	4.975	6.53	26.56
1290	778.6	923.6	80.66	5.287	5.957	22.65
1291	740.8	NA	73.39	6.185	6.134	20.17
1292	1043.4	NA	77.81	6.567	6.61	22.75
1300	NA	154.4	NA	4.162	6.472	19.74

Accession	Score apr2008	Score jun2008	Score apr2009	Score jun2010	Regrowth jul2009	Homogeneity 2008
1001	5.219	6.571	5.731	5	4.001	4.405
1003	9.084	7.624	4.354	3.5	6.288	0.395
1004	7.496	7.59	3.435	3	4.823	2.116
1005	7.328	7.002	3.858	3	5.321	4.345
1007	10.239	9.14	4.336	3.5	5.573	1.954
1008	8.351	6.625	4.654	4	5.967	4.977
1009	9.42	9.121	4.598	5	4.61	2.291
1012	7.805	8.602	2.35	2.667	5.128	0.879
1013	7.863	7.088	3.965	3.333	4.74	2.092
1016	4.414	5.408	3.485	5.5	3.085	1.007
1017	7.441	6.471	4.189	3.667	5.349	2.148
1018	7.237	7.087	5.286	3	6.238	3.277
1019	9.099	8.423	6.332	6.333	5.523	1.05
1020	4.655	4.85	3.052	3.5	4.681	1.439
1024	8.217	6.694	4.876	4.5	5.34	1.906
1026	6.016	6.724	5.216	4	6.331	2.392
1028	5.94	5.502	5.005	2.5	5.356	1.729
1035	4.745	6.865	5.434	5	6.479	3.473
1036	4.034	4.596	3.226	3.5	3.255	1.107
1040	9.462	8.177	5.471	5	6.309	3.807
1041	7.734	7.894	4.918	5	6.437	1.372
1042	10.064	8.701	4.369	2	6.199	1.324
1043	9.036	8.556	4.765	4.5	6.049	0.662
1044	9.181	9.19	6.507	6	6.796	-0.357
1045	8.612	6.627	4.738	4.5	5.548	1.923
1046	8.666	8.57	4.774	4	5.98	2.046
1071	8.152	7.26	3.926	4.667	3.391	2.583
1072	6.667	5.628	2.303	3.5	2.555	2.307
1077	6.489	7.737	3.479	2.5	3.696	0.174
1095	4.065	4.974	5.118	3.5	5.847	3.563
1100	6.754	7.288	4.178	1.5	4.931	3.284
1101	6.518	5.553	2.932	2.5	5.495	2.105
1102	7.121	5.086	3.448	2	5.793	6.257
1103	7.186	6.535	4.6	4.5	4.587	2.674
1104	7.784	9.311	4.038	3	5.331	1.581

1105	7.033	6.263	3.585	1.5	3.753	3.458
1106	7.008	6.074	3.494	2	5.697	3.552
1107	6.693	7.476	4.73	3	4.373	1.073
1108	7.515	5.705	3.351	3	4.971	2.65
1110	5.902	7.671	2.791	3	4.731	1.899
1111	7.274	6.342	4.599	2.5	5.361	6.92
1112	6.528	5.478	3.562	2.5	3.427	1.074
1113	6.453	6.656	4.529	3.333	5.979	3.354
1114	6.912	5.601	3.538	2.5	4.369	2.876
1115	7.5	8.18	6.255	5.333	6.523	0.657
1116	6.845	4.891	3.162	1.667	4.06	3.952
1117	6.667	6.823	3.998	2.5	4.736	2.9
1118	6.772	6.413	4.545	2	6.629	2.628
1119	6.621	8.045	3.676	2	4.688	0.907
1120	6.082	5.735	3.723	2	4.962	3.092
1121	6.033	5.727	3.791	2.333	3.676	0.804
1122	5.935	4.863	4.444	2.5	5.034	2.238
1123	6.349	5.569	3.476	2	4.199	3.39
1124	7.256	5.769	3.067	2.5	3.743	3.929
1125	8.584	6.91	4.226	3	6.082	1.946
1127	7.36	7.865	3.894	3.5	5.097	3.353
1128	7.707	6.711	2.469	2	5.156	3.742
1129	5.779	5.053	2.406	1	3.5	1.759
1131	4.942	5.059	3.872	3	5.902	3.974
1132	8.309	7.686	3.152	2.5	4.118	2.54
1133	8.467	8.217	4.853	3	5.871	2.23
1134	7.822	7.592	3.092	1	4.425	2.747
1136	5.995	6.525	4.47	2	4.832	1.503
1137	3.441	6.102	4.165	3.5	6.279	1.533
1138	5.296	4.852	5.403	3.5	5.366	3.876
1139	4.542	4.996	4.95	3.5	3.836	1.569
1140	5.772	5.403	4.013	4	2.211	3.482
1141	7.758	7.75	4.163	3	5.697	2.642
1142	7.678	6.412	4.251	1.5	5.217	0.651
1143	6.09	6.726	4.187	2.5	6.074	2.104
1145	8.37	8.633	4.976	3.5	6.078	3.675
1148	5.832	6.789	5.015	4.5	6.722	1.974

1151	7	6.889	3.897	2.5	4.792	1.953
1152	4.873	7.477	3.257	2.5	4.634	2.339
1153	4.281	7.144	4.075	4	5.172	1.396
1154	5.54	7.489	4.297	3.5	6.017	4.117
1155	6.552	8.951	4.478	4	5.718	2.526
1156	5.848	6.815	4.538	4	5.196	3.492
1157	5.339	6.738	3.637	3.333	4.93	2.526
1158	6.345	7.667	3.866	4	5.46	1.655
1159	5.997	7.171	4.221	4	4.746	2.403
1160	6.419	6.16	3.986	5	4.922	4.708
1161	5.908	6.523	4.652	4.667	5.58	2.182
1163	6.354	7.302	4.401	2	3.72	2.973
1164	6.072	7.668	4.13	3	5.777	3.768
1165	4.789	4.001	5.185	5.333	3.469	4.016
1166	6.249	7.083	3.98	3	4.626	3.022
1167	6.056	7.167	4.602	3.5	6.057	1.556
1168	5.709	6.471	4.429	3.5	4.551	2.039
1169	7.016	5.08	3.88	4	5.257	2.571
1170	6.999	6.7	4.337	2.5	4.329	3.944
1171	7.859	NA	3.807	2	5.419	-0.108
1172	5.002	8.107	5.055	3.5	5.6	0.784
1173	4.958	7.932	5.969	4.5	5.26	2.785
1174	6.196	7.369	4.429	5.667	5.272	3.028
1175	4.931	7.853	3.048	3.5	3.744	0.841
1176	3.679	6.739	3.806	3.5	4.918	1.536
1177	4.887	6.486	3.009	3.667	3.406	1.938
1179	5.211	4.296	1.519	3.5	2.075	0.642
1180	5.804	7.479	4.431	3.5	5.115	6.075
1181	6.032	7.27	5.091	4	6.517	1.919
1183	4.724	5.28	3.925	3.667	5.334	4.585
1184	2.97	5.691	2.208	3	1.549	2.779
1185	5.626	4.185	2.186	3.5	2.092	2.342
1187	6.109	8.05	4.604	2	4.981	3.595
1188	6.732	7.862	4.562	3	3.658	6.51
1189	6.65	7.654	3.652	1.5	5.154	3.136
1190	6.054	7.906	4.163	3.667	5.804	4.26
1191	6.389	8.213	5.075	5	5.172	3.356

1193	4.485	8.59	4.16	3.5	5.287	1.269
1194	4.787	6.978	4.51	4	5.419	3.299
1195	6.035	7.45	6.419	4.5	6.392	4.346
1196	8.049	7.837	4.784	5	5.596	3.54
1197	7.566	7.872	3.977	3.5	5.037	2.828
1198	6.421	4.6	4.164	3	5.713	1.383
1199	6.392	7.686	3.686	2.667	5.171	0.994
1200	5.783	5.438	5.373	4.5	5.617	4.984
1201	6.124	6.899	4.194	3	5.921	3.061
1202	7.169	9.517	3.711	2	4.597	2.363
1203	7.321	8.553	4.782	7	5.389	2.566
1204	6.865	4.57	4.422	4.5	5.43	3.681
1205	7.227	8.798	4.096	2	4.482	2.512
1206	6.209	7.366	5.072	3.5	4.004	3.261
1207	7.023	8.377	3.997	3	3.957	1.244
1209	6.733	8.107	3.211	2	4.036	1.834
1210	6.43	7.356	4.917	4.5	4.656	3.005
1211	7.263	6.707	3.913	3.5	5.09	NA
1212	5.998	6.967	3.695	3	5.826	2.883
1213	6.403	6.835	5.834	4.667	6.897	4.35
1214	5.462	6.069	3.737	2.333	4.525	4.145
1218	6.965	6.552	3.112	2.5	3.807	6.199
1219	6.05	8.567	3.488	3	3.469	5.646
1220	7.14	7.585	2.559	1.5	4.587	2.77
1221	5.019	7.594	3.107	2	4.625	2.094
1228	6.054	6.76	3.887	4	4.989	1.333
1230	7.407	7.341	6.705	4.667	6.745	2.024
1231	5.291	7.488	5.603	4.5	5.811	3.912
1233	2.956	5.514	3.836	4	6.37	3.366
1241	2.333	7.102	3.327	2	5.722	1.83
1245	3.534	6.52	3.652	1.5	3.96	1.558
1247	5.156	6.399	2.735	3	3.971	0.511
1248	3.241	8.054	4.337	2.5	5.233	0.348
1249	2.014	8.35	2.693	3	5.312	1.151
1250	3.56	6.436	2.985	4	4.061	2.713
1252	5.613	7.882	3.126	3.5	4.293	2.216
1253	4.848	7.151	3.512	2.667	4.544	2.39

1254	6.44	4.66	3.752	5.5	5.127	2.528
1256	5.942	6.017	1.517	3	1.392	1.882
1258	5.076	7.522	4.002	3	5.248	3.16
1259	4.651	7.221	3.799	2.667	5.623	2.855
1260	6.993	7.727	3.819	3.333	5.286	NA
1261	5.47	6.277	4.008	3.5	6.484	3.562
1262	7.504	7.167	4.384	5.667	3.346	2.365
1263	7.944	7.772	4.536	5	5.626	1.683
1264	7.961	7.87	4.244	3.333	4.36	0.205
1265	6.055	6.442	4.695	4	4.482	1.821
1266	9.383	9.32	5.883	4.667	4.564	0.584
1289	4.681	4.5	3.987	6.5	1.65	1.361
1290	4.761	7.739	6.112	5.5	6.021	3.147
1291	4.758	7.791	5.745	5.5	5.596	2.239
1292	4.323	7.121	3.763	4	5.605	0.995
1300	4.44	NA	2.744	4	3.119	NA

APPENDIX 4: PREDICTED MEANS OF SELECTED ACCESSIONS (CHAPTER 2) USING REML ANALYSIS FOR MORPHOLOGICAL EVALUATIONS

Accession	Habit 2008	Habit 2009	Leaf length 2009	Inflorescence length 2009	Leaf number per stem 2009	Inflorescence number per stem
1001	3.208	3.111	18.22	9.11	11	8.333
1005	NA	4	15.83	8.28	11	7
1007	3.526	3.889	17	11	12.56	8.111
1012	3.297	3.889	15.72	13.67	11.11	6.667
1013	3.022	3.961	14.3	7.06	10.03	4.937
1017	3.421	3.367	14.36	8.96	9.68	6.542
1018	NA	3.556	16.44	9.33	11.11	6.444
1019	NA	3.778	16.78	11.28	10.56	7.222
1026	2.887	3.778	16.44	10.28	11.89	6.889
1028	2.891	3.778	14.94	9.28	10.67	8.556
1041	3.25	3.556	15.28	12.83	9.89	6.333
1043	NA	3.556	18.83	12.61	13.22	9.889
1071	2.806	3.367	15.45	8.92	9.41	5.819
1077	3.516	3.667	16.39	13.11	11	8.222
1103	3.274	3.556	15.67	10.78	11.89	9.333
1104	3.683	3.534	16.7	12.13	12.52	9.375
1110	3.243	4	17.83	11.78	10.44	6.778
1113	3.306	3.778	16.56	10.5	11	8.222
1118	3.336	3.778	17.44	9	10.11	7.444
1123	2.832	3.556	14.61	9.51	9.79	6.507
1127	NA	3.556	15.33	10.66	11.47	7.972
1140	2.797	2.933	13.19	6.82	6.97	5.916
1156	2.794	3.556	15.89	12.28	11.78	8.889
1157	3.462	3.444	15.17	10.79	9.85	7.097
1163	NA	3.303	14.83	10.14	10.27	7.494
1165	3.113	3.556	15.39	8.83	10.44	8.778
1169	2.269	3.444	16	11.72	9.56	6.667
1171	3.426	3.383	14.18	11.47	11.12	8.341
1179	1.873	1	13.22	8.67	7.67	5.111
1197	NA	3.556	16.39	11.11	10.33	7.444
1199	3.581	3.333	17.17	11.65	10.93	7.793
1200	2.798	3.667	16.39	9.22	10.11	8
1210	3.002	3.444	14.28	11.33	9.56	7.222
1211	NA	3.222	14.11	9.22	10.6	6.972
1213	2.29	3.444	17.17	10.94	10.11	7.333
1220	3.551	3.778	15.67	12.33	10.89	9.556
1230	2.668	3.556	16.22	9.44	12.56	8.667

1241	2.859	3.933	16.94	11.14	9.52	6.454
1245	3.409	3	14	10.94	14.22	14
1253	3.384	3.778	17.72	11.6	11.47	8.597
1256	2.942	2.333	18.56	14.07	10.64	10.749
1259	3.377	4	16.83	8.78	12.1	8.472
1260	NA	3.667	18.83	11.39	11.67	7.667
1261	3.382	3.933	15.78	10.65	11.3	7.749
1262	3.239	3.333	15.56	7.17	9.78	7.444
1264	2.843	3.367	16.57	12.38	11.35	7.459
1266	3.507	3.778	16.56	12.33	10.67	6.556
1290	3.307	3.766	15.19	10.82	9.8	7.416

Accession	Leaflet number per leaf 2009	Ratio leaflet length leaflet width	Stem thickness 2008	Stem thickness 2009	Stem number per plant 2009	Stem length 2009
1001	27.11	3.675	5.376	3.167	24.56	75.89
1005	27	4.032	2.799	3.667	26.67	76.11
1007	18.67	3.596	4.217	3.111	18.67	95.89
1012	22.22	3.945	4.159	3.611	20	102
1013	28.14	3.773	4.735	3.013	28	79.67
1017	23.5	3.622	4.437	3.141	30.75	87.75
1018	23.56	3.568	5.345	4.278	23.89	96.89
1019	21.22	3.514	3.186	3.778	34.33	83.56
1026	22.67	3.461	7.209	3.778	22.56	98
1028	28	3.812	3.376	3.833	28.78	91.78
1041	22.11	3.266	6.135	3.111	24.44	92
1043	22.11	3.64	3.04	4.222	17.11	102.89
1071	25.33	3.958	4.488	2.891	27.73	75.94
1077	23.78	3.191	4.423	3.167	17.56	99.78
1103	19.56	2.954	3.352	3.111	17.33	89.33
1104	19.58	2.771	4.645	3.35	21	94.33
1110	18.89	3.36	NA	2.778	17.78	78.33
1113	24.44	3.176	5.639	3.722	26.11	85.67
1118	20.89	3.468	4.525	3.333	27.67	95.11
1123	20.33	3.645	3.676	2.547	21.43	76.94
1127	21.33	3.082	3.751	2.989	20.13	89.85
1140	21	3.442	3.67	2.384	47.66	61.5
1156	22.33	3.314	3.547	3.389	26.89	90.33
1157	21	3.392	5.017	3.052	16.63	85.35
1163	24.14	4.19	4.678	3.353	22.15	90.25
1165	23.78	3.424	3.776	3.222	26.89	74.44
1169	24.22	2.715	4.82	3.5	18.89	80.22
1171	22	3.193	3.62	3.061	22.5	82.86
1179	24.56	3.517	4.189	1.778	46.11	59.33
1197	23.67	3.256	4.245	3.333	23	87.44
1199	24.67	3.555	5.015	3.404	23.14	88.65
1200	23.89	3.271	4.2	3.111	26	80.89
1210	24.22	3.616	6.012	3.222	29.67	81.11
1211	25.67	3.803	NA	3.239	27.25	82.72
1213	22	2.803	3.603	3.667	24.11	83.89
1220	19.67	2.944	4.237	3.444	18.44	91

1230	20	3.203	4.698	3.556	27.22	95.56
1241	20	4.018	4.406	3.043	29.19	91.36
1245	23.67	3.903	3.99	3.167	16.44	80.44
1253	20.33	3.261	5.524	3.364	21.5	94.1
1256	20.11	3.263	3.296	2.384	18.33	86.5
1259	20.33	3.49	4.295	2.864	16.13	94.85
1260	19.22	3.416	NA	3.222	17.44	89.56
1261	21.33	3.388	5.003	3.217	21.99	92
1262	27.89	3.712	4.963	3.056	31.22	77.44
1264	20.33	3.483	5.434	3.516	20.5	94.58
1266	23.44	3.341	6.405	3.444	28.89	104.44
1290	22.66	3.216	3.181	3.467	19.16	102.5

Accession	Dominant flower colour 2008	Dominant flower colour 2009	Dominant stem colour 2008	Dominant stem colour 2009	Dominant leaf colour 2008
1001	4.981	3.152	1.431	2.111	5.198
1005	5.044	2.306	0.932	2	5.461
1007	3	2.163	2.012	2.444	7.014
1012	5.999	1.701	2.189	4.111	5.134
1013	4.448	3.166	1.813	1.143	6.059
1017	5.029	2.227	1.892	3.25	6.189
1018	5.321	2.334	1.073	2	4.621
1019	5	1.531	1.021	3.111	7.159
1026	3	2.163	0.805	2.111	5.995
1028	5.001	2.662	0.778	1.333	4.559
1041	5.131	1.974	2.371	1.444	6.768
1043	3.428	0.924	1.007	2.556	6.701
1071	5.624	2.141	1.511	4.25	5.407
1077	3.998	1.692	2.28	2.778	4.232
1103	5	1.571	4.169	3.667	5.777
1104	4.793	2.807	0.199	3.5	7.132
1110	6.001	1.725	0.81	3	6.246
1113	4.844	1.174	1.135	1.111	5.682
1118	4.369	1.744	1.747	2.333	5.232
1123	4.963	1.543	1.019	2.667	5.556
1127	5.834	2.518	1.163	2.778	5.249
1140	4.972	1.915	1.954	1.667	3.651
1156	3.974	2.281	2.094	3.667	5.812
1157	4.517	1.103	2.206	3.667	5.955
1163	4.792	1.875	1.112	2	5.347
1165	4.503	2.592	2.243	4.111	5.819
1169	4.258	2.387	2.797	4.333	6.356
1171	5.892	2.213	3.686	4.625	5.959
1179	6.219	3.067	2.915	7.333	6.208
1197	5.001	1.79	1.101	5.111	7.164
1199	5.015	2.439	2.078	4.444	6.747
1200	4	1.97	1.242	1.222	6.157
1210	5.12	2.316	2.148	2.556	5.826
1211	NA	1.457	NA	2.667	NA
1213	3	1.594	7.282	3.444	6.888

1220	2.825	1.789	1.888	2.111	4.915
1230	5.002	1.421	1.216	1.111	5.948
1241	4.03	2.385	0.742	1	6.252
1245	3.625	1.102	2.516	6.444	5.169
1253	6.014	1.817	2.085	2.667	7.257
1256	7.131	3.122	1.633	3.111	3.098
1259	3.817	1.776	1.933	4.556	5.357
1260	NA	2.083	NA	3.444	NA
1261	5.229	2.063	2.14	2.167	7.04
1262	5.604	2.682	1.947	1.667	5.566
1264	2.954	1.673	2.363	2.917	7.125
1266	4.656	1.711	2.485	1.667	5.775
1290	6	1.868	0.801	3	4.989

APPENDIX 5: PREDICTED MEANS USING REML ANALYSIS FOR SEED MORPHOMETRIC EVALUATIONS ON 75 SELECTED ACCESSIONS FOR SEED PRODUCTION

accession	Area cm²	Length	Width	Width/ Length	Shape	Shape change
1001	0.26391	7.06942	5.18896	0.736432	0.674797	0.0983425
1003	0.271567	7.37228	4.94732	0.679187	0.722507	0.0592457
1005	0.306899	7.73557	5.5964	0.727519	0.667484	0.0924039
1007	0.251776	7.42821	4.77592	0.652242	0.688853	0.0981249
1008	0.260096	6.89639	5.27444	0.766877	0.717072	0.0432008
1013	0.290657	7.95903	5.30624	0.674345	0.632999	0.155286
1018	0.23526	6.93029	4.80774	0.709092	0.725097	0.0943902
1019	0.281113	7.24803	5.18818	0.720466	0.759054	0.0424073
1040	0.241491	6.68913	4.76074	0.713877	0.772286	0.0299226
1042	0.243874	6.82716	4.80214	0.706656	0.747907	0.0319968
1043	0.238381	6.57846	4.8482	0.741154	0.766526	0.0315002
1044	0.212047	6.70385	4.48179	0.684008	0.715131	0.115602
1045	0.234394	6.82834	4.72315	0.697862	0.719176	0.0654414
1046	0.231283	6.72548	4.80516	0.71723	0.710763	0.0476673
1071	0.295049	7.51286	5.44325	0.728389	0.682475	0.0897499
1077	0.218811	6.34393	4.63955	0.73312	0.759848	0.0279334
1100	0.283661	7.44163	5.20549	0.702215	0.719408	0.0500119
1102	0.236782	6.66183	4.82863	0.730885	0.755499	0.052859
1103	0.24738	6.84487	4.80645	0.704911	0.7571	0.0316014
1104	0.239109	6.70266	4.6939	0.701691	0.76582	0.0285947
1105	0.285151	7.58681	5.17994	0.690007	0.717554	0.0640278
1106	0.260895	7.06413	4.93785	0.700294	0.752486	0.0294902
1108	0.262989	7.25707	4.91962	0.678925	0.727583	0.0411607
1110	0.248008	6.54998	5.13208	0.784683	0.774884	0.0281445
1111	0.271193	7.21039	5.12411	0.712259	0.73363	0.0534151
1112	0.223557	6.35884	4.67985	0.738564	0.774986	0.0260534
1113	0.275602	7.29076	5.03246	0.693943	0.751782	0.0271642
1114	0.260652	7.16579	4.88311	0.686862	0.73502	0.0503056
1115	0.271144	7.13679	5.14161	0.723741	0.739663	0.0549018
1116	0.192542	5.96925	4.42049	0.743431	0.749764	0.036481
1117	0.247463	7.13114	5.05508	0.718085	0.634241	0.180241
1118	0.239396	6.86904	4.71496	0.689253	0.727011	0.0404814
1119	0.258278	7.24086	4.90505	0.680712	0.701824	0.0558037
1120	0.249229	7.11024	4.79625	0.6801	0.717348	0.0673227
1121	0.300678	7.56144	5.22551	0.692669	0.744966	0.0342042
1123	0.306111	7.61105	5.55886	0.733761	0.72575	0.0353667
1127	0.230296	6.84295	4.60224	0.678656	0.731288	0.0610678
1128	0.254463	6.86035	4.98689	0.731507	0.765482	0.0289267
1132	0.371483	8.47852	6.11313	0.723883	0.655053	0.120872

1133	0.291018	7.45562	5.21069	0.701812	0.753291	0.027495
1134	0.277231	7.14483	5.197	0.730831	0.771985	0.0258462
1141	0.255425	7.06283	4.85522	0.689938	0.746615	0.0250277
1142	0.243261	6.84129	4.82538	0.706531	0.724259	0.0319645
1145	0.28761	7.87534	5.10535	0.658791	0.698696	0.0894332
1155	0.203116	6.2512	4.33293	0.695353	0.762585	0.0283768
1156	0.210173	6.31927	4.45676	0.707471	0.781511	0.0277158
1163	0.232327	6.44621	4.79943	0.745546	0.771541	0.0231336
1164	0.227198	6.47549	4.60926	0.713683	0.774902	0.0264288
1169	0.230385	6.61318	4.69504	0.713678	0.74922	0.0357552
1170	0.216382	6.3358	4.56708	0.724208	0.770625	0.0321687
1171	0.277362	7.32335	5.06971	0.695558	0.727523	0.0469073
1187	0.262468	6.97196	5.01063	0.721264	0.753986	0.0333018
1188	0.24282	6.94248	4.83343	0.697978	0.706522	0.0626888
1189	0.295649	7.52924	5.29587	0.704963	0.738398	0.0289006
1190	0.275297	7.2017	5.11158	0.712639	0.739267	0.0419207
1196	0.233428	6.65024	4.77717	0.71984	0.742255	0.0345213
1197	0.226003	6.63021	4.65281	0.704045	0.744699	0.0377088
1198	0.271241	7.12701	5.03964	0.711418	0.753741	0.0407154
1199	0.261679	6.96005	5.02151	0.722707	0.76545	0.0267149
1200	0.235938	6.64306	4.80294	0.725667	0.745597	0.031406
1201	0.233769	6.56214	4.7967	0.734974	0.765503	0.0302405
1202	0.244024	6.82807	4.84549	0.712015	0.731473	0.0445491
1204	0.234977	6.69696	4.75886	0.71204	0.74234	0.0382935
1205	0.273248	7.06079	5.0839	0.721628	0.767586	0.0237572
1207	0.280294	7.11346	5.57466	0.785498	0.67264	0.121561
1209	0.242301	6.58291	4.87285	0.741256	0.786933	0.0183441
1210	0.265379	7.01842	5.09058	0.72797	0.706511	0.0696444
1211	0.267163	6.98118	5.20486	0.749172	0.756338	0.0318914
1212	0.215376	6.13296	4.71451	0.771876	0.784941	0.0258487
1213	0.244707	6.6845	4.85314	0.729025	0.780472	0.0241125
1214	0.21938	6.3787	4.59478	0.721382	0.775215	0.0332751
1218	0.250492	7.04098	4.94096	0.705645	0.659208	0.101487
1220	0.246654	6.82463	4.81939	0.708509	0.772401	0.0251096
1230	0.232443	6.61811	4.68176	0.713017	0.767864	0.0303004
1260	0.260497	7.03132	4.88663	0.699361	0.753935	0.026373

APPENDIX 6: PAPER ACCEPTED FOR PUBLICATION IN PLANT GENETIC RESOURCES, 2011

ISSN 1479-2621
Plant Genetic Resources: Characterization and Utilization (2011) **9**(1); 70–85
doi:10.1017/S1479262110000328

Sainfoin (*Onobrychis viciifolia*): a beneficial forage legume

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Abstract

The *Onobrychis* genus comprises a few agronomically important forage legume species, with sainfoin (*Onobrychis viciifolia*) being the most widespread. *O. viciifolia* has a long history of traditional culture worldwide, but its use has declined in western countries over the last decades. It suffers from low productivity and is more difficult to maintain than other legumes but is known to have valuable characteristics such as palatability and drought tolerance. Recent studies suggest that it has several other highly beneficial properties due to its unique tannin and polyphenol composition. Condensed tannins present in *Onobrychis* species have been shown to confer anthelmintic properties, increase protein utilization and prevent bloating; they may also have the potential to reduce greenhouse gas emissions. Positive effects on wildlife and honey production could also be advantageous in the context of sustainable farming. Modern breeding programmes have not been a priority, leading to a lack of genetic knowledge in comparison to extensively used forage legumes. It is expected that potential for *O. viciifolia* improvements could be achieved by rigorous characterization of the available germplasm and utilization of characters derived from close relatives of the genus. Breeding priorities for the future would include enhanced germination and improved early establishment, allied to the best anthelmintic properties observed in some varieties.

Keywords: anthelmintic properties; forage legume; greenhouse gas emissions; *Onobrychis viciifolia* (sainfoin); sustainable agriculture; tannin protein utilization

Introduction

Sainfoin (*Onobrychis viciifolia*) is an excellent forage legume, which was grown in Europe before the widespread use of commercial fertilizers. In many parts of Europe, the cultivation of forage legumes has decreased, especially in the 1980s, when the impact of support payments from the Common Agricultural Policy (CAP) was to favour intensive production. Following CAP reforms in 2005, a single farm payment was introduced, which is uncoupled from production volumes but linked to environmental, food safety and animal welfare standards. The new policy aims to make European Union (EU) farmers more competitive. Higher levels of inorganic nitrogen fertilizers suit high-yielding grass *Trifolium* sp. mixtures. This trend is now changing, and pressure to reduce energy consumption and environmental pollution and to improve agricultural sustainability is driving lower input agronomy. The cost of inputs, especially nitrogen and phosphate, has more than doubled in the past 5 years, and farmers are reconsidering the use of forage legumes, which are better suited to low input regimes. Forage legumes have been shown to increase the efficiency of nitrogen use and reduced nitrogen

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transit from the soil. Moreover, global warming is projected to increase the yield of forage legumes, relative to grasses, due to a combination of their relative responses to heat, light and nutrient sequestration (Haynes, 1980; Clarke *et al*., 2000).

The *Onobrychis* genus belongs to the *Fabaceae* family and *Hedysareae* tribe. It is widespread in temperate zones of North America, Europe and Middle East. *O. viciifolia* is of significant agricultural use as a perennial forage and fodder legume. *O. viciifolia* tolerates drought, cold and low nutrient status. These properties make it very popular on Middle East plateaus and some areas of Spain, Italy and Eastern Europe. In Europe, cultivation of *O. viciifolia* has also suffered from increased competition from higher yielding forages (mostly *Medicago sativa* and *Trifolium* sp.).

Recent research works have highlighted several additional beneficial properties of *O. viciifolia* for livestock, and this is mainly due to the nature of its particular secondary metabolites. Furthermore, it is known to enhance diversity and stability of agroecosystems, representing a valuable pollen and nectar source for honey production. *O. viciifolia* and related species would benefit from characterization and development to fully exploit these properties. Rigorous taxonomic characterization is limited and sometimes contradictory. Very little has been done in terms of either molecular genetics or cytological characterization, which is crucial to initiate modern breeding programmes. Realization of the potential of *O. viciifolia* is limited by a number of issues including low productivity, erratic establishment and variability in the presence of beneficial phytochemicals in different genetic lines.

Taxonomy

O. viciifolia belongs to the genus *Onobrychis*, which belongs to the tribe *Hedysareae* of the subfamily *Papilionoideae* of the *Fabaceae* family (previously *Leguminosae*). Many contradictions are found in the taxonomy of *Onobrychis,* mostly due to the different approaches in species delimitation, resulting in a varying number of recognized species (Emre *et al*., 2007). Yildiz *et al*. (1999) suggested that the genus *Onobrychis* comprises about 170 species, based on fruit morphology. They are classified into two subgenera, *Sisyrosema* and *Onobrychis*, and eight sections. Guner *et al*. (2000) estimated that there are 54 species of *Onobrychis* divided into five sections. Širjaev (1925) produced a useful classification, which is presented in Table 1. *O. viciifolia* is the most widespread species (Celiktas *et al*., 2006) for which several synonyms are used in the literature: *Hedysarum onobrychis* L., *Onobrychis sativa* Lam., *Onobrychis viciaefolia* Scop. and *O. viciifolia* Scop. Sánchez-Yélamo (2006) characterized a subset of the genus using isozyme methods; section Eubrychis was clustered in a main group, while taxa in the subsections *Hispanicae, Brachysemiae* and *Macropterae* appear differentiated from the subsection *Vulgatae*. Using a classification based on seed protein profiles, Emre *et al*. (2007) showed species of sections Lophobrychis, Onobrychis and Hymenobrychis clustered together. More recently, Ahangarian *et al*. (2007) noted that subgenus *Sisyrosemae* seems to be derived from subgenus *Onobrychis* based on intervening transcribed sequences of the nuclear ribosomal DNA.

Table 1. Classification of the *Onobrychis* genus adapted from Širjaev (1925)

Sections under each subgenus of *Onobrychis*	
Euonobrychis = *Onobrychis*	*Sisyrosemae*
Dendrobrychis	Anthyllium
Lophobrychis	Afghanicae
Hemicyclobrychis	Heliobrychis
Eubrychis = Onobrychis	Hymenobrychis

Botanical description of *O. viciifolia*

O. viciifolia is an erect or suberect plant, from 40 to 100 cm in height (Frame *et al*., 1998). Many hollow stems, arising from basal buds, form a branched crown. Each stem has pinnate leaves formed with 10–28 leaflets grouped in pairs on long petioles and with a terminal leaflet. The stipules are broad and finely pointed. The inflorescences develop on axillary tillers with about 80 pinkish red, or rarely white, melliferous flowers (Fig. 1). Each flower can produce a kidney-shaped seed contained in a brown pod. The fruit is either spiny or spineless. The degree of spininess is characteristic for different lines and is genetically determined (Thomson, 1951b). The size of the true seeds is variable from 2.5 to 4.5 mm long, 2 to 3.5 mm broad and 1.5 to 2 mm thick (Fig. 2). Unmilled seed and milled seed weigh approximately 24 and 15 g/1000 numbers, respectively. The fruit colour is mainly determined by the ripeness at harvesting time. A deep taproot with a few main branches and numerous fine lateral roots forms the root system.

O. viciifolia is divided into two agricultural types. The 'common' type (*O. sativa* var. *communis* Ahlefed) is from central Europe and remains prostrate in the year of sowing. It is also named single-cut *O. viciifolia* because regrowth after the first spring cut is only vegetative. The giant type or double-cut *O. viciifolia* (*O. sativa* var. *bifera* Hort.) is from the Middle East and reflowers after being cut (Badoux, 1965). The giant type has proportionally less stem per plant, longer stems and more

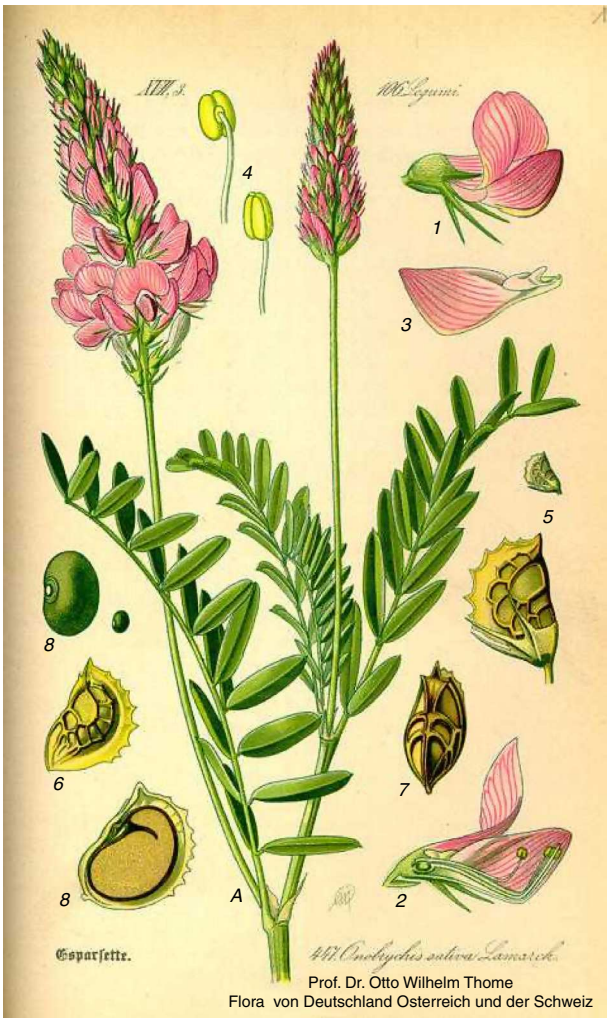

Fig. 1. *Onobrychis viciifolia.* (with kind permission from K. Stueber at http://www.biolib.de/) (A colour version of this figure can be found online at journals.cambridge.org/pgr).

internodes per stem. It also has more leaflets per leaf than the common type (Thomson, 1951a). Otherwise, they are very similar with respect to seed weight, colour and spininess of the unmilled fruit (Thomson, 1951b). Negri and Cenci (1988) characterized 20 populations of *O. viciifolia* from central Italy and noted morphological differences according to altitude. High altitude led to populations with reduced dimensions; leaflets had a round shape, prostrate growth habit and shorter peduncle of inflorescence but a greater length of inflorescence.

Cytological aspects

O. viciifolia is reported to be either a diploid or a tetraploid species with respectively $2n = 2x = 14$ and $2n = 4x = 28$ chromosomes (Frame *et al.*, 1998). However, Abou-El-Enain (2002) discusses the occurrence of series of $2n = 22$, 27, 28 and 29 chromosomes ($2n = 3x + 1$, $4x$-1, $4x$ and $4x + 1$), which demonstrates the role of aneuploid alteration from the chromosome number based on multiples of $x = 7$ in the evolution of this species. Most literature, though, only refers to the tetraploid type (Negri *et al.*, 1987; Kidambi *et al.*, 1990; Tamas, 2006), with an average chromosome length of 3.39 μm. A recent study has confirmed that diploid types exist but are very rare (Hayot *et al.*, unpublished) (Fig. 3).

Breeding system

O. viciifolia is an outbreeding insect-pollinated species. A range of insect species successfully pollinate flowers, but the most important are *Apis mellifera* (honey bee), *Bombus* sp. (bumble bees) and, to a lesser extent, *Osmia* (solitary). Although it is possible to self-pollinate sainfoin plants under controlled conditions, resultant plants lack vigour and produce few if any viable seeds (Beat Boller, pers. commun.). *Onobrychis* along with many other members of the *Fabaceae* is considered to be an obligate insect-pollinated species (Hanley *et al.*, 2008).

History of cultivation

O. viciifolia has been cultivated for hundreds of years in many parts of the world, including Asia, Europe and

Fig. 2. *Onobrychis viciifolia* seed variability: 1208, 1292, 1257 and 1126 correspond to different accessions collected at the National Institute of Agricultural Botany, Cambridge UK CB30LE (A colour version of this figure can be found online at journals.cambridge.org/pgr).

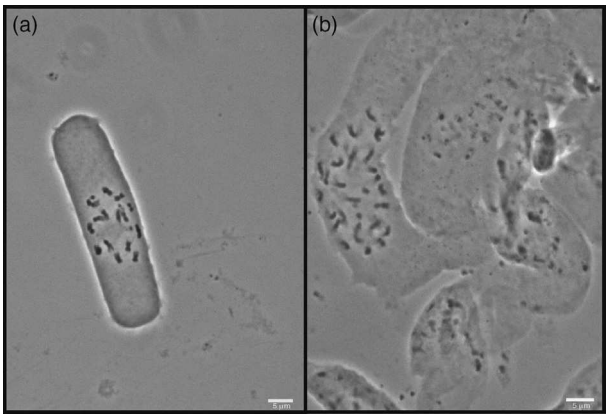

Fig. 3. Metaphasic *O. viciifolia* meristematic root cell, (a) diploid with $2n = 2x = 14$ and (b) tetraploid with $2n = 4x = 28$.

North America (Frame *et al.*, 1998). One in seven fields in southern England were covered in pink flowers until the mid 1940s. Cotswold Seeds Ltd. hold English covenants from the 1800s, stating that tenant farmers were required to grow *O. viciifolia* to maintain soil fertility. Farmers considered it as the 'best cog in the farming wheel' for sustainable farming, improving soil fertility of poor chalky soils covering parts of southern England. A once-popular rotation in Hampshire in the 1830s consisted of a 4-year *O. viciifolia* ley followed by wheat, turnips and spring barley.

O. viciifolia is native to South Central Asia and was introduced into central Europe in the 15th century (Burton and Curley, 1968). It was first cultivated in Southern France in 1582, following which it spread across Europe (Piper, 1924) and into North America by 1786. It was being cultivated in the UK by the mid 17th century (Hartlib, 1652) and gained popularity in many areas of Britain where it was used to feed the heavy horses, and the aftermath (leafy stubble) was used for grazing lambs (Koivisto and Lane, 2001). Today, it is still popular in Eastern Europe, Italy, Spain, Iran and, especially, Turkey where about 94,000 ha were grown in 2001 (Eken *et al.*, 2004). Elsewhere, over the last 40 years, *O. viciifolia* has experienced a constant decline in Europe (Borreani *et al.*, 2003). It is recorded that more than 150 tonnes of seeds were sold every year in the late 1950s in the UK, enough for 2500 ha (Hill, 1997). In the late 1970s, only approximately 150 ha were cropped. Today, *O. viciifolia* has become rare in the UK, and this is due, in part, to its poor response to the changing requirements and circumstance of British agriculture (Hutchinson, 1965). Rochon *et al.* (2004) pointed out that the decline of forage legumes in Europe has been due to the farmers support payments towards intensive production using cheap inorganic fertilizers since the early 1970s, together with the expansion and dominance of autumn cereal cropping from the 1960s (Hill, 1997). In Italy, Borreani *et al.* (2003) noted that structural changes, allied to the gradual disappearance of livestock farms in hilly areas, may have contributed, especially the reduction in draught horses (Newman, 1997), for which it was a major feed. Finally, agronomic limitations have contributed to its decline, including low yield, low persistence and poor regrowth after the first cut, compared with *M. sativa* (Sims *et al.*, 1968; Borreani *et al.*, 2003).

Agronomy

Climate and soil requirements

O. viciifolia grows in a wide range of climatic conditions in Europe, North America, Asia, Australia and New Zealand, in neutral and alkaline soils of pH 6 or above, in dryland and irrigated areas. In the UK, it has always been linked with calcareous chalky or limestone soil (Frame *et al.*, 1998) and is intolerant of water logging (Sheldrick *et al.*, 1987). Only a thin and patchy sward grew on clay soil at pH 6 with failures on alluvial sand at or below pH 5 in the Thames Valley (Bland, 1971). Doyle *et al.* (1984) estimated that *O. viciifolia* could potentially be grown on 950,000 ha in England and Wales, where the soil is sufficiently alkaline. Meyer and Badaruddin (2001) compared the frost tolerance of young seedlings of several legume species; *O. viciifolia* seedlings were more resistant than *M. sativa* and most of the *Trifolium* species. Only *Trifolium hybridum* seedlings were more resistant. Although there is little published data, there is considerable observational evidence that *O. viciifolia* is tolerant of relatively high temperatures; in 2009 and 2010, it was grown in small plots in northern Greece and southern Spain, where temperatures of >32°C were often recorded (Ioannis Hadjigeorgiou, pers. commun., Agricultural University of Athens).

Sowing and weed control

O. viciifolia seeds are sold in two forms, 'unmilled' fruit containing a singe seed and cleaned, 'milled seed' (Thomson, 1951b). Authors disagree as to which perform better in terms of germination (Wiesner *et al.*, 1968; Chen, 1992). In the UK, *O. viciifolia* is normally drilled between April and July when the soil is warm enough for rapid germination and when there is sufficient moisture. A seed rate of 7 kg/ha and a row spacing of 60 cm are recommended for seed production (or 40 kg/ha × 15 cm for hay production) (Goplen *et al.*, 1991). An optimal plant density of 100 plants/m^2

produced the maximum yield of 62.5 kg/ha in a greenhouse study (Sheehy *et al.*, 1984).

O. viciifolia is usually considered to be a non-aggressive crop with slow regrowth after cutting; therefore, weed competition needs to be minimized at establishment; in a study by Moyer (1985), weeds formed 98% of the biomass in the absence of herbicides during the first year. Establishment is improved by drilling in combination with *Festuca pratensis* or *Phleum pratense* or by undersowing with spring barley. A mixture with *Lotus corniculatus* was also effective (Cooper, 1972). A small range of herbicides can be used including [4-(2-methyl-4-chlorophenoxy) acetic acid)] for broad leaves weeds and [4-(2-methyl-4-chlorophenoxy) butyric acid] at the first trifoliate stage. Carbetamide [(R)-1-(ethylcarbamoyl) ethylcarbanilate] maintains swards during the winter (Sheldrick and Thomson, 1982; Frame *et al.*, 1998). In addition to aiding establishment, grass and *O. viciifolia* mixtures yield more than each component separately (Dubbs, 1968; Frame *et al.*, 1998; Koivisto and Lane, 2001). Liu *et al.* (2006) recommended a rate of 2:1 of *O. viciifolia* to *F. pratensis*.

Symbioses

Symbiotic interactions occur between Gram-negative *Rhizobiaceae* and legume plant roots. In the resultant nodules, differentiated bacteroides use a nitrogenase enzyme complex to reduce atmospheric nitrogen to ammonia, which is subsequently converted to amino acids in the plant. In return, the rhizobia receive products of photosynthesis. The interaction shows a degree of specificity and is dependent upon a reciprocal molecular dialogue between the host plant and the rhizobia (Sprent, 2003). Bacteria from the genera *Mesorhizobium*, *Rhizobium* and *Bradyrhizobium* all interact with *Onobrychis* (Baimiev *et al.*, 2007). Unlike some leguminous species, *O. viciifolia* can be cross-inoculated by *Rhizobium* species from several other host plant species, including *Hedysarum* sp., *Coranilla* sp., *Dalea purpurea, Dalea candida, Astragalus alpinus*, *Oxytropis maydelliana* and *Oxytropis arctobia*. (Burton and Curley, 1968; Prévost *et al.*, 1987).

Onobrychis forms arbuscular mycorrhizas (AM), which is a symbiosis between plant roots and fungi. It is one of the most widespread symbiotic associations found in plants and, unlike nodulation, is relatively non-specific, highly compatible and long lasting. The endophytes are primarily from the genus *Glomus*; they access carbon products from photosynthesis, while the fungus increases sequestration of mineral nutrients, especially phosphate from the soil through the extensive mycelium (Barea and Azcon-Aguilar, 1983; Harrison, 1998). AM can also improve nitrogen fixation, through phosphate supply and uptake from the soil (Barea *et al.*, 1987).

Nitrogen fixation and fertilization

Overall, nitrogen fixation rates of *O. viciifolia* have been measured to be within the range of other forage legumes (Liu, 2006). The rate of nitrogen fixation in *O. viciifolia* nodules has been described as 'sometimes insufficient', and nitrogen deficiency symptoms can be seen in inoculated plants (Burton and Curley, 1968; Sims *et al.*, 1968). This may be associated with energy supply; *O. viciifolia* required gross photosynthesis of 258 kg carbohydrate/ha/d compared with 234 kg carbohydrate/ha/d for *M. sativa* (Sheehy and Popple, 1981).

In comparison to other legumes, the nitrogen fixation rate of *O. viciifolia* has been measured in terms of both the amount of nitrogen fixed and expressed in terms of resultant increase in yield. For *O. viciifolia,* the rate in most situations was between 130 and 160 kg/ha, compared with 140 and 210 kg/ha for *M. sativa*; this resulted in an increase in yield of 17 and 25%, respectively (Provorov and Tikhonovich, 2003). Upper limits in a nitrogen-free situation were higher, at 270 and 550 kg/ha. However, these data should be viewed with caution, since neither the *O. viciifolia* variety, nor the rhizobial identity was specified. In another study by Prévost *et al.* (1897), 47 different rhizobial strains were assessed with a good cultivar, Melrose, in a nitrogen-free, pot experiment. The impact of the resultant symbiosis varied from 'ineffective' in terms of growth response to 'high'. Numerically, the range was from 8 to 140 mg total nitrogen/pot. The authors concluded that plant growth is dependent on an effective symbiosis, but that several strains of rhizobia were unable to meet nitrogen requirements. They further noted that under their experimental conditions, all inoculated plants showed symptoms of nitrogen deficiency at early growth stages, but that with 'effective' strains, these symptoms disappeared with time.

In general, nitrate fertilization is known to reduce nodulation and nitrogen fixation rates of legumes (Hartwig and Nosberger, 1996). However, low levels of inorganic nitrogen stimulate nitrogen fixation in *O. viciifolia*, with consequent biomass production increasing by 20–30% (Koter, 1965; Sims *et al.*, 1968, 1975). This yield relationship was not, however, observed with more fertile soil conditions and may be variety dependent. Badoux (1965) reported a 4% reduction after a 90 kg/ha/year treatment with giant *O. viciifolia*. Bland (1971) reported that *O. viciifolia* responded well to farmyard manure, phosphate and potash; Sheehy *et al.* (1984) noted that *O. viciifolia* required more P_2O_5 and NO_3 than *M. sativa* but less K_2O and $CaCO_3$.

A number of other studies are somewhat contradictory in terms of absolute amounts, probably due to differences in soil fertility, but generally agree that moderate phosphate and potash, together with low levels of nitrogen, increase productivity and stand persistence (Meyer, 1975; Shan *et al.*, 1991; Tufenkci *et al.*, 2006). In an extension of the study of comparative rhizobial effectiveness, Prévost *et al.* (1897) considered the effectiveness of the best strains in the presence of low levels of NO_3–N. They found that at early stages of growth, even the best rhizobial strains had no significant effect on shoot or root dry matter, but at later growth stages, up to 68% of nitrogen in the plant was derived from fixation, and that at harvest, yield was significantly higher than both controls and less-effective strains. In conclusion, it seems likely that *O. viciifolia* is dependent on some mineral nitrogen at early growth stages, regardless of rhizobial identity. Later growth stages will significantly benefit from an effective symbiosis.

Forage and fodder characteristics

O. viciifolia in the UK is traditionally used as a hay crop, although it can be cut for silage (Bland, 1971; Sheldrick *et al.*, 1987). The leafy 'stubble' can be used for light grazing, but only in the late autumn, to allow the crop time to replenish root reserves (Sheldrick *et al.*, 1987). Depending upon the growing conditions, *O. viciifolia* will yield between 7 and 15 tonnes/ha dry matter, which was *c.* 20% lower than *M. sativa*. This was due to a lower leaf area index, a more prostrate canopy structure and less efficient nitrogen fixation (Frame *et al.*, 1998). Regrowth is slow, and it is important to allow enough time to replenish root reserves to maintain its persistence and longevity.

Seed production

O. viciifolia is generally regarded as an outbreeding species, with a self-incompatibility system (Tasei, 1984). Negri *et al.* (1987) suggests that the system may not be strict, and that self-fertilization can occur; however, recent studies in Switzerland showed that selfing rates are very low (Beat Boller, pers. commun.). Honey bees (*A. mellifera*) and leafcutting bees (*Megachile rotundata*) are efficient pollinators (Goplen *et al.*, 1991). During peak bloom in June–July, which takes about 60 d, it is recommended that optimal seed yield requires two to three colonies of honey bees or 20,000 leafcutting bees per hectare. Authors do not, however, agree on the optimal requirement for pollinators; it has been suggested that 20 hives were not sufficient for bee saturation on 2.4 ha (Dubbs, 1968). *O. viciifolia* produces seeds on an inflorescence consisting of 5–80 flowers, each of which can produce one seed, although seed set rarely exceeds 55%. A plant may produce 5–40 tillers, each with 3–5 inflorescences. The number of flowers per inflorescence, inflorescences per tiller and tillers per plant are a function of interrelated environmental and genetic factors (Carleton and Wiesner, 1968). Seed size is inversely proportional to the number of seeds per head (Carleton and Wiesner, 1968). Seed yield per hectare is generally 500–900 kg of clean seeds, but yields of 1100 kg/ha have been obtained with some cultivars in Canada (Goplen *et al.*, 1991). Seed longevity is maximized by storing them in the unmilled state (Thomson, 1952).

Pest and diseases

O. viciifolia is relatively free from serious pest and disease problems compared with other legumes (Goplen *et al.*, 1991). In the UK, root, crown and stem rot caused by *Sclerotinia trifoliorum* occurs (Hughes, 1949), but crown and root rot caused mainly by *Fusarium solani* is probably the most important factor affecting longevity. Wilt caused by *Verticillium* can also be a problem in the UK and Germany. Stem and leaf diseases include leaf spot (*Ramularia onobrychidis* and *Septoria orobina*), ring spot (*Pleospora herbarum*), leaf and stem spot (*Ascochyta onobrychidis*), rust (*Uromyces onobrychidis*), chocolate spot (*Botrytis conerea*) and powdery mildew (*Erysiphe polygoni*). Powdery blight (*Ascochyta fabae*) has been reported in Iran and Turkey.

Root-feeding insects can make establishment of new stands difficult and reduce the longevity of established stands. Adult *Sitona scissifrons* weevils become active in the field in June and eat the edges of the leaves leaving characteristic notches along the leaves. This damage could be disastrous at the seedling stage in the field (Wallace, 1968). Their larvae feed on the roots, and this reduces the persistence of *O. viciifolia* plants because pathogens invade the root scars (Morrill *et al.*, 1998). Other members of *Sitona* (*S. lineata*, *S. calloso* and *S. crinita*) have damaged *O. viciifolia* in Europe (Wallace, 1968). Larvae of a clearwing moth, *Sesia chalcidiformis*, feed also with roots of *O. viciifolia* in Europe (Wallace, 1968). Some other insect species can damage the stems and leaves of *O. viciifolia,* but most of them cause only minor damage. These include sugarbeet webworm (*Loxostege similalis* and *L. sticticalis*) and larvae of *Colias eurytheme*, *C. edusa, C. hyale, Phytonomus farinosus* and *Hypera trilineata* (Wallace, 1968). Sucking insects damage the stems, leaves and in some case the developing seeds, especially the potato leafhopper (*Empoasca*

fabae). *Lygus elisus*, *L. hesperus* and *Adelphocoris lineolatus* feed on buds, flowers and seeds (Morrill *et al.*, 1998).

A number of insect species damage seed production in Europe. The *O. viciifolia* midge (*Contarinia onobrychidis*) is a serious pest in some parts of Europe, particularly in England. The larvae form galls in the flower heads, and the seeds fail to develop (Wallace, 1968). *Eurytoma onobrychidis*, the *O. viciifolia* seed chalcid, is also a serious pest in some areas of Europe (Wallace, 1968). Other insects can also damage seed production in Europe but are less aggressive; these include *Perrisia onobrychidis*, *Apion pisi*, *Odontothrips intermedius*, *Otiorhynchus ligustici* and *Melanotus erythropus*. Seed production in the USA is decreased by a bruchid, *Bruchidius unicolor*, and *Bruchophagous* spp., a seed-infesting insect. The root-knot nematode (*Meloidogyne* spp.) and the stem and bulb nematode (*Ditylenchus dipsaci*) have both been found on *O. viciifolia* in the USA (Mathre, 1968).

Beneficial aspects

Food source for bees and other pollinators

The decline in wild and managed pollinators in the UK, Europe, the USA and parts of Asia has been widely reported (Biesmeijer *et al.*, 2006; Cox-Foster *et al.*, 2007; Williams and Osborne, 2009; Potts *et al.*, 2010). A single definitive cause has not been identified, but the consensus among many bee keepers is that several factors are involved, with nutrition being one of them (MAAREC, 2006; Van Engelsdorp *et al.*, 2007). The agricultural trend towards monoculture may exacerbate pollinator decline because pollen derived from a single source can compromise nutrition and health (Hendrikx *et al.*, 2009; Aston *et al.*, 2009). *O. viciifolia* flowers are a rich source of pollen and nectar, attracting ten times more bees than *Trifolium repens* (Rosov, 1952; McGregor, 1976; Kells, 2001) and are visited by managed and indigenous pollinator insect species, including *Apis*, *Bombus* and *Osmia* (Horne, 1995; Clement *et al.*, 2006; Howes, 2007; USDA SARE, 2007; Westphal *et al.*, 2008; Taki *et al.*, 2009). Rozen *et al.* (2010) noted that *O. viciifolia* is the sole pollen source for *Osmia avosetta* bees in Turkey, which build elaborate colourful nests from its pink petals. In the UK, *O. viciifolia* starts flowering in May and continues for about 60 d. The crops can be cut to give 2–3 flowering periods, which continue until early September. They could thus provide a good source of pollen and nectar for over-wintering bees (Manning, 2001; Tasei and Aupinel, 2008; Manning, 2006; Eischen *et al.*, 2009). Recent research by Syngenta (2008) recommended the general sowing of *O. viciifolia* to enable bees to lay down food reserves for the winter. It has been estimated that *O. viciifolia* yields up to 400 kg/ha of honey (Howes, 2007).

Animal feed and nutritional benefits

As long ago as the 16th century, Olivier de Serres described a forage called *sainfoin* in France and *herba medica* in Italy and referred to 'the inordinate praise the plant has been given, for its medical virtues and for fattening the livestock that graze on it…' (http://en.wikipedia.org/wiki/Sainfoin). It is attractive to both wild and domesticated animals, including elk, deer, sheep, goats, cattle and horses (http://plants.usda.gov/plantguide/doc/pg_onvi.doc). The Greek term *Onobrychis* signifies that it is 'keenly eaten by donkeys' (http://www.pedigreequery.com/sainfoin).

The voluntary intake of *O. viciifolia* by sheep and cattle is 20–24% higher than for grasses and 10–29% higher than for red clover or *M. sativa* (Waghorn *et al.*, 1990; Karnezos *et al.*, 1994). As a result, *O. viciifolia* supports high growth rates in young ruminants (Thomson *et al.*, 1971; Parker and Moss, 1981; Marten *et al.*, 1987; Hart and Sahlu, 1993). Ruminants can safely consume large amounts because it does not cause bloat, which can occur when forages such as *Trifolium* sp., *M. sativa* or young grass are fermented rapidly in the rumen, thus generating a stable foam that traps the fermentation gases (McMahon *et al.*, 2000; Waghorn and McNabb, 2003). The expanding rumen puts pressure on vital organs, and this can be fatal if not tackled in time.

It is a well-established fact that tannins are the active compounds that prevent bloating; stands of *O. viciifolia, Lotus* or mixtures containing these crops and wild species such as dock (*Rumex obtusifolius)* (Li *et al.*, 1996) contain suitable proportions of the tannins. *O. viciifolia–M. sativa* mixtures have also proved bloat-safe (McMahon *et al.*, 2000; Mueller-Harvey, 2009; Wang *et al.*, 2006). This bioactivity of tannins (Fig. 4) is attributed to their capacity to inhibit the growth of *Streptococcus bovis*, a rumen bacterium that produces dextran-slime, and their ability to destabilize the proteinaceous foam in the rumen (Jones *et al.*, 1994; Waghorn and McNabb, 2003). Relatively low tannin concentrations in plants are sufficient to remove the danger of bloating following ingestion (1–5 mg tannins/g dry matter) (Li *et al.*, 1996).

Nutritive value

Animals fed on *O. viciifolia* make large body weight gains, >400 g/d for goats and lambs, and the literature relating to ruminants has been summarized by Waghorn (2008). In trials, there were a range of responses to

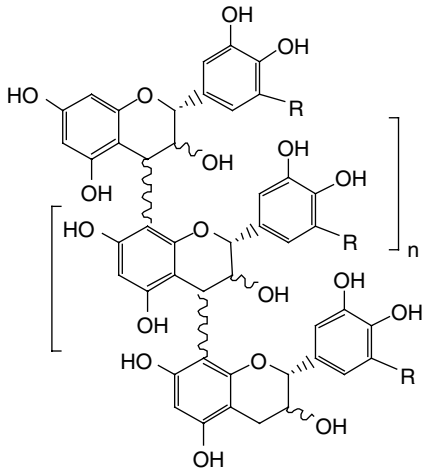

Fig. 4. *Onobrychis viciifolia* tannins.

material, and this is undoubtedly complicated by varietal differences. For tannin-containing legumes and tannin-free *M. sativa*, *T. repens* and pasture, three trials recorded 19–24% higher daily lamb and cow gains on *O. viciifolia* than *M. sativa*, while two trials recorded 3% lower lamb gains on *O. viciifolia* than *T. repens*. Comparing sheep responses to *O. viciifolia*, white clover and lucerne, Waghorn *et al.* (1990) calculated that *O. viciifolia* tannins caused from 19 to 124% more nitrogen to be retained and from 17 to 56% more nitrogen to be digested in the small intestine. This is due to a more efficient utilization of the metabolizable energy and protein in *O. viciifolia* (Thomson, 1982; Hart and Sahlu, 1993; Mueller-Harvey, 2009). As a result, ruminants retained between 2.6 and 4.8 g/d more nitrogen from *O. viciifolia* (if harvested early) than *T. repens* or *M. sativa* (Egan & Ulyatt, 1980). John and Lancashire (1981) also found that live weight gains by sheep revealed the following relative feeding values: white clover (100), *O. viciifolia* (97*), Lotus pedunculatus* (87), lucerne (78) and *Trifolium pratense* (78). A similar trend was found for young goats grazing on *O. viciifolia* or *M. sativa* (Hart and Sahlu, 1993). Some of these benefits were observed despite the fact that the crude protein (CP) content of *O. viciifolia* was less than that of *M. sativa*. Scharenberg *et al.* (2007) measured 10–21% higher plasma levels ($P < 0.001$) of essential amino acids when feeding *O. viciifolia*, which has previously been noted for *L. corniculatus* tannins (Waghorn, 2008). In addition, the organic matter and nitrogen had from 6 to 7% higher levels of digestibility when wethers were fed with ensiled *O. viciifolia–M. sativa* mixtures compared with *M. sativa* (Wang *et al.*, 2007). The optimal *O. viciifolia:M. sativa* ratio for ensiling and ruminal fermentation was found to be 4:6. These benefits have been found in both fresh and conserved *O. viciifolia* (Waghorn *et al.*, 1990; Hill, 1997). *O. viciifolia* silage has up to 50% less soluble non-protein nitrogen and 53% less free amino acid contents than *M. sativa* silage (Albrecht and Muck, 1991). Ruminants make inefficient use of nitrogen from grass and *M. sativa* silages for milk and meat synthesis (Tamminga, 1992; Givens and Rulquin, 2004). In the absence of tannins, nitrogen fractions can be extensively hydrolysed during ensilage and are subsequently rapidly degraded in the rumen. To achieve the average milk yield of UK dairy cows (5800 kg/lactation), a cow needs to consume at least 160 kg nitrogen annually. Of this, 70% or more (>110 kg nitrogen) is excreted in faeces and urine. However, plant tannins reduce the degradation of proteins during fermentation in the silo or rumen, and this enables ruminants to benefit from a better amino acid supply. Tannins exert this protective effect by binding to plant proteins; the resulting complex is less liable to microbial degradation; this process has been described as 'rumen-escape protein' (Mueller-Harvey, 2006).

Most binding by tannins takes place at the isoelectric point of the protein (Jones and Mangan, 1977). In the case of rubisco, which is the major protein in green plants, complex formation is favoured at a ruminal pH of *c.* 5.6–6.8. The pH in the digestive organs postruminally ranges from <3 (abomasum) to *c.* 8 in the lower intestines. At these low or high pH values, tannin–protein complexes are easily dissociated, and protein becomes available for enzymatic hydrolysis (Jones and Mangan, 1977). Therefore, when less of the protein is digested in the rumen, more can be hydrolysed postruminally into amino acids, which are then available to the animal via absorption from the intestines (Scharenbergy *et al.,* 2007; Waghorn, 2008). Enhanced amino acid absorption has also been demonstrated for other tanniferous species such as *L. corniculatus* (Waghorn, 2008). Absorption of essential amino acids increased by 62%, while milk, meat and wool yields, ovulation rate and lambing percentage all increased by 10–15% (Waghorn *et al.*, 1990; Min *et al.*, 2003; Waghorn, 2008). Grabber *et al.* (2002) estimated that if *M. sativa* contained these types of tannins, it could save $300 million in the USA. The EU 'LEGGRAZE' project has already demonstrated that the use of *O. viciifolia* and *L. corniculatus* significantly increased CP intakes (g/d) compared with *T. repens*, *T. incarnatum* and *T. ambiguum*, but not compared with *M. sativa*; sheep performance (live weight gains) paralleled these results (Molle *et al.*, 2008).

Unlike other tannin-containing species, the tannin-filled cells in *O. viciifolia* are evenly distributed

throughout the plant in all organs except the roots (Lees *et al.*, 1993). The effects of tannins on protein solubilization and degradation appear to be highly localized in plant tissues (Min *et al.*, 2000); therefore, this even distribution may facilitate a rapid reaction between plant proteins and tannins during mastication and fermentation in the rumen or silo. Furthermore, *O. viciifolia* has low levels of endogenous plant proteases, which are less than half that of *M. sativa* (Kingston-Smith *et al.*, 2003). These enzymes are involved in the early stages of protein degradation in the rumen (autolysis), thus potentially contributing to the rumen escape mechanism. Recent research within the EU 'Healthy Hay' project demonstrated that *O. viciifolia* has considerable peroxidase activity (Ahmad *et al.*, 2010), which may contribute to the formation of covalent tannin–protein links during drying or ensiling, further reducing ruminal protein degradation.

It is important, however, to recognize that there are some less favourable reports about the nutritive value of *O. viciifolia,* which showed that it did not affect nitrogen retention or amino-acid absorption (Fraser *et al.*, 2000; Bermingham *et al.*, 2001; Scharenberg *et al.*, 2008). By using polyethylene glycol, which has a strong affinity for tannins, it is possible to ascertain whether tannins modify the protein digestion process. Parker and Moss (1981) and Karnezos *et al.* (1994) did not find any differences between *O. viciifolia* and *M. sativa*, whether grazed or fed as hay, in terms of weight gain of heifers or lambs. In another study, Aufrère *et al.* (2008) did not find any difference in terms of nitrogen utilization by sheep between fresh *O. viciifolia* and *M. sativa*. Although lambs retained the same amount of nitrogen from *O. viciifolia* and *M. sativa* silages (Fraser *et al.*, 2000), the CP content of *O. viciifolia* was lower than that of *M. sativa* at 121 and 183 g protein/kg dry matter, respectively.

Potential environmental benefits

Rising costs of nitrogen fertilizers is driving a trend towards more sustainable farming methods; a home-grown protein source is therefore becoming more important (Pecetti *et al.*, 2009). Furthermore, nitrogen balance studies consistently show that *O. viciifolia* reduces urinary nitrogen and increase faecal nitrogen excretions (Aufrère *et al.*, 2008; Mueller-Harvey, 2009; Theodoridou *et al.*, 2010). Once urinary nitrogen is in the environment, it is rapidly converted to N_2O, a potent greenhouse gas (Tamminga *et al.*, 2007). Conversely, faecal nitrogen is an environmentally safer form (Grabber *et al.*, 2002). Studies have also indicated that tannins decreased methane production (g/kg dry matter intake) *in vivo* in sheep and goats by between 20 and 55% (Tamminga *et al.*, 2007; Waghorn, 2008). In addition, evidence has been presented that *O. viciifolia* can also reduce the shedding of *Escherichia coli* O157:H7 in cattle faeces (Berard *et al.*, 2009). This is a particular problem during the spring thaw in Canada, when manure mixes with the snowmelt and contaminates the environment in the run-off from the frozen soil.

Biohydrogenation for improved milk and meat composition

Methane production and biohydrogenation are closely linked as both the processes remove hydrogen from the rumen fermentation system (Tamminga *et al.*, 2007). However, biohydrogenation destroys potentially valuable plant compounds such as polyunsaturated fatty acids (PUFAs), which are beneficial to human health if they can be transferred from the plant into meat or milk (Givens and Shingfield, 2004; Tamminga *et al.*, 2007). Biohydrogenation converts PUFAs into the less desirable saturated fatty acids.

Anti-parasitic properties

Gastrointestinal nematodes are a major, worldwide threat to animal welfare and production (Hoste *et al.*, 2006). Nematode resistance against all three classes of anthelmintic drugs is developing rapidly across the world, challenging conventional drug treatments and threatening areas of livestock farming. FAO guidelines strongly recommend that combined strategies should be developed to deal with this problem (FAO, 2004). Annual ruminant production losses due to parasitic nematodes cost millions of dollars (>US$ 300 m in USA in 1995; ~AU$ 220 m in Australia; ~US$ 26 m in Kenya) (FAO, 2004; Waller, 2006). Alternative sustainable solutions are now urgently required to replace these drugs. Tannins represent an untapped, natural resource of biologically active compounds. They can modulate nematode biology at key life cycle stages. *O. viciifolia* and other tannin-containing forage legumes have potential for reducing worm burdens in ruminants (Waghorn, 2008). Both dried and ensiled *O. viciifolia* lowered faecal egg counts from lambs that were infected with *Haemonchus contortus* nematodes (Häring *et al.*, 2008) and *Trichostrongylus colubriformis* (Rios-de Álvarez *et al.*, 2008). Similar results were obtained with goats infected with *H. contortus*, *Teladorsagia circumcincta* and *T. colubriformis* (Paolini *et al.*, 2005). In addition, enhanced immune cell development was observed in the intestinal tissue from sheep after feeding *O. viciifolia* (Rios-de Álvarez

et al., 2008). Regular feeding of *O. viciifolia* hay to grazing lambs and goats could, therefore, be used to improve host resilience and thus lower pasture contamination. The anthelmintic bioactivity of *O. viciifolia* is maintained in hay or silage (Ojeda-Robertos *et al.*, 2010), thus providing an early spring resource around parturition when host immunity of mother and newborn is low.

Phytochemical basis for nutritional and veterinary benefits

The phytochemical composition of *O. viciifolia* has been investigated for over 35 years (Bate-Smith, 1975; Dewick, 1977; Ingham, 1978; Russell *et al.*, 1984; Koupai-Abyazani *et al.*, 1992, 1993a,b; Lu *et al.*, 2000; Marais *et al.*, 2000; Regos *et al.*, 2009). While most research indicated that the *L. pedunculatus* and *O. viciifolia* tannin structures were similar in terms of procyanidin to prodelphinidin ratios (Fig. 4) (Czochanska *et al.*, 1980; Marais *et al.*, 2000; Hedqvist *et al.*, 2000), other reports suggested that *O. viciifolia* tannins were unusual, difficult to extract (Bate-Smith, 1975), with very high molecular weights (Jones *et al.*, 1976) and capacities for binding proteins (Jones *et al.*, 1976; McAllister *et al.*, 2005). An optimal tannin concentration has yet to be defined for *O. viciifolia*. Studies on *Lotus* species showed that dietary tannin concentrations below 5% (on a dry matter basis) would benefit ruminant production, while higher levels inhibited protein and carbohydrate digestions (Barry and McNabb, 1999; McMahon *et al.*, 2000; Min *et al.*, 2003). Conversely, *O. viciifolia* containing up to 8% tannins had a high nutritive value for sheep (Waghorn and McNabb, 2003).

Recent research in the EU 'Healthy Hay' project revealed that the tannin composition in the National Institute of Agricultural Botany *O. viciifolia* germplasm collection showed considerable variation (Stringano *et al.*, 2010), varying by more than fourfold. The average number of flavanol units per tannin polymer (i.e. mean degree of polymerization) varied sevenfold, prodelphinidin tannin constituents ranged from 53 to 95% and flavanol *trans*:*cis* ratios ranged from 12:88 to 34:66. The protein-binding strengths of these molecules are still to be determined. Given this variation among the different *O. viciifolia* lines, it is not surprising that many animal-feeding trials showed contradictory results in terms of enhancing nitrogen absorption by ruminants (see above). Most research did not specify which *O. viciifolia* lines had been used to feed the animals, and this now precludes any further interpretation of the results.

The analytical techniques may not have been appropriate for nutritionally relevant tannins (Mueller-Harvey, 2006). *O. viciifolia* tannins are difficult to purify, and this complicates their analysis (Gea *et al.*, 2011). Stewart *et al.* (2000) demonstrated that the widely used HCl–butanol assay can also lead to contradictory results if inappropriate and impure tannin standards are used. We consider that the excellent nutritional properties of *O. viciifolia*, which were reported by several authors, were caused by particularly effective tannins or enzymes, and their identities await further investigation through a multidisciplinary approach. We also propose that *O. viciifolia* composition can be optimized through plant breeding.

Breeding and varieties

Various breeding programmes have successfully improved the agronomic performance of both *M. sativa* and *Trifolium* species, but little research has been directed towards improving *O. viciifolia* varieties in Europe. A few isolated breeders still register new cultivars adapted for specific needs, but the breeding programmes are very small and do not take into account the huge diversity available. *O. viciifolia* varieties differ largely in terms of winter hardiness, maturity, yield potential and many other factors (Shaw, 1968). Agricultural varieties of *O. viciifolia* do not rigidly align with either of the two original types, common or giant. Some well-known landraces are *Cotswold Common*, *Hampshire Common* and *Somborne,* which are primarily common types, while *Hampshire Giant* and *English Giant* are giant types. New varieties derived from these two types include *Nova* and *Melrose* developed in Canada in the 1970s, *Eski*, *Remont* and *Remunex* from the USA in the 1960s and 1970s, *Zeus* and *Vala* from Italy, *Perly* from Switzerland, *Fakir* from France and *Emyr* from Hungary (Koivisto and Lane, 2001). Shoshone was released in 2006 in Wyoming, USA and has good agronomic performances (Gray *et al.*, 2006). *G35* was released in New Zealand and is adapted to New Zealand climatic conditions (Rumball and Claydon, 2005). Some breeding is also currently ongoing in Italy (Martiniello, 2005). In 2010, only 19 varieties of *O. viciifolia* were registered (http://ec.europa.eu/food/plant/propagation/catalogues/comcat_agri_2008/37.html) on the European common catalogue. There are no *O. viciifolia* guidelines available for the conduct of tests for distinctness, uniformity or stability produced by the International Union for the Protection of New Varieties of Plants. Furthermore, the biological potential of the lines is still not taken into account in breeding programmes.

Conclusion and future perspectives

O. viciifolia is potentially a very useful forage crop, particularly for sustainable farming approaches. It represents

an alternative to *M. sativa* or *Trifolium* sp. in some locations and can be fed *ad libitum* in contrast to other forage legumes. The beneficial effects of *Onobrychis* sp. could be realized by rigorous modern breeding inputs. Currently, a 4-year European project 'Healthy Hay' supported by the European Commission and consisting of a consortium of 14 partners is evaluating agronomic, genetic, nutritional and veterinary properties from a unique and extensive germplasm collection. Other *Onobrychis* species that could be used to increase the diversity available for breeding are currently being identified. In addition, other species of *Onobrychis* that may be crossed with *O. viciifolia* to enhance biological properties and agronomic potential are being considered for selection (Hayot *et al.*, unpublished). Taxonomic clarification, identification of beneficial compounds and their metabolic pathways, biological properties assessment and selection of the more promising lines and species are among the information expected to arise from this project. These will be an important source for future *O. viciifolia* breeding programmes. A precedent has been set in New Zealand, where breeding programmes have already improved the persistence of *L. corniculatus*. (Waghorn, 2008).

The divergent findings in terms of the efficiency of nitrogen utilization by ruminants may reflect the choice of lines that were used. Unfortunately, variety name, the type (common or giant) or even plant maturity were rarely specified. We now know that these factors and location all affect tannin and polyphenol composition (Theodoridou *et al.*, 2010). Aufrère *et al.* (2008) concluded that further research was needed to determine qualitative and quantitative changes in tannin contents and structures according to growth stages and the implications of these changes for tannin–protein interactions in the digestive tract.

Different lines with contrasting polyphenol, tannin and enzyme compositions also need to be evaluated for their nutritional and anthelmintic efficacy to provide guidelines for future plant breeding programmes. One breeding goal will be to develop varieties with stable, heritable tannin and polyphenol composition (Mueller-Harvey and Dhanoa, 1991).

Future plant breeding priorities

In the future, breeding priorities should include an improvement in both total germination and synchronicity of germination in a sward. This has a negative impact on early establishment and can lead to weediness during the first year, which causes an unwelcome management problem to farmers. Other aspects such as a slightly reduced root to shoot ratio of early establishment could also be improved during seedling establishment. Most genetic lines currently channel resources into a very long taproot at the expense of leafy growth; this ensures that the plant is highly drought tolerant, but the lack of a leafy canopy makes it prone to competition from weeds during this first year. These priorities should be allied to the best anthelmintic properties observed in some varieties, due to the presence of the tannins and other secondary metabolites.

Acknowledgements

We acknowledge the support from the EU (MRTN-CT-2006-035805; 'Healthy Hay' project) and thank Dr Franck Carbonero for his critical support.

References

Abou-El-Enain MM (2002) Chromosomal criteria and their phylogenetic implications in the genus *Onobrychis* Mill. sect. Lophobrychis (Leguminosae), with special reference to Egyptian species. *Botanical Journal of the Linnean Society* 139: 409–414.

Ahangarian S, Kazempour Osaloo Sh and Maassoumi AA (2007) Molecular phylogeny of the tribe Hedysareae with special reference to *Onobrychis* (Fabaceae) as inferred from nrDNA ITS sequences. *Iran Journal of Botany* 13: 64–74.

Ahmad AF, Thill J, Regos I, Hayot C, Halbwirth H, Smith LMJ, Treutter D and Stich K (2010) Biosynthesis of flavonoids in sainfoin (*Onobrychis viciifolia*). Poster presented at the 25th International Conference on Polyphenols, 23–27 August 2010, Montpellier, France.

Albrecht KA and Muck RE (1991) Proteolysis in ensiled forage legumes that vary in tannin concentration. *Crop Science* 31: 464–469.

Aston D, Carreck N, Davis I, Lovett T and Metcalf P (2009) British Beekeepers' Association. Honey bee health – research concepts. http://www.britishbee.org.uk/files/bbka-research-concepts.pdf

Aufrère J, Dudilieu M and Poncet C (2008) *In vivo* and *in situ* measurements of the digestive characteristics of sainfoin in comparison with lucerne fed to sheep as fresh forages at two growth stages and as hay. *Animal* 2: 1331–1339.

Badoux S (1965) Etude des caracteres morphologiques, physiologiques et agronomiques de populations d'esparcette (*Onobrychis* spp.). *Recherche Agronomique Suisse* 4: 111–190.

Baimiev AK, Baimiev AK, Gubaidullin II, Kulikovo OL and Chemeris AV (2007) Bacteria closely related to *Phyllobacterium trifolii* according to their 16S rRNA gene are discovered in the nodules of Hungarian sainfoin. *Russian Journal of Genetics* 43: 587–590.

Barea JM and Azcon-Aguilar C (1983) Mycorrhizas and their significance in nodulating nitrogen-fixing plants. *Advances in Agronomy* 36: 1–54.

Barea JM, Azconaguilar C and Azcon R (1987) Vesicular-arbuscular mycorrhiza improve both symbiotic N2 fixation and N uptake from soil as assessed with a N15 technique under field conditions. *New Phytologist* 106: 717–725.

Barry TN and McNabb WC (1999) The implications of condensed tannins on the nutritive value of temperate forages fed to ruminants. *British Journal of Nutrition* 81: 263–272.

Bate-Smith EC (1975) Phytochemistry of proanthocyanidins. *Phytochemistry* 14: 1107–1113.

Berard NC, Holley RA, McAllister TA, Ominski KH, Wittenberg KM, Bouchard KS, Bouchard JJ and Krause DO (2009) Potential to reduce *Escherichia coli* shedding in cattle feces by using sainfoin (*Onobrychis viciifolia*) forage, tested *in vitro* and *in vivo*. *Applied and Environmental Microbiology* 75: 1074–1079.

Bermingham EN, Hutchinson KJ, Revell DK, Brookes IM and McNabb WC (2001) The effect of condensed tannins in sainfoin (*Onobrychis viciifolia*) and sulla (*Hedysarum coronarium*) on digestion of amino acids in sheep. *Proceedings of the New Zealand Society of Animal Production* 61: 116–119.

Biesmeijer JC, Roberts SP, Reemer M, Ohlemueller R, Edwards M, Peeters T, Schaffers A, Potts SG, Kleukers R, Thomas CD, Settele J and Kunin WE (2006) Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science* 313: 351–354.

Bland BF (1971) *Crop Production: Cereals and Legumes*. Academic Press: London UK, pp. 431–444.

Borreani G, Peiretti PG and Tabacco E (2003) Evolution of yield and quality of sainfoin (*Onobrychis viciifolia* Scop.) in the spring growth cycle. *Agronomie* 23: 193–201.

Burton JC and Curley RL (1968) Nodulation and nitrogen fixation in sainfoin (*Onobrychis sativa* LAM.) as influenced by strains of rhizobia. *Sainfoin Symposium*: 3–5.

Carleton AE and Wiesner LE (1968) Production of sainfoin seed. *Sainfoin Symposium, Montana State University*, December 12–13 1968.

Celiktas N, Can E, Hatipoglu R and Avci S (2006) Somatic embryogenesis, callus production, and plantlet growth in sainfoin (*Onobrychis viciifolia* Scop.). *New Zealand Journal of Agricultural Research* 49: 383–388.

Chen B (1992) Sainfoin. In: Lanzhou (ed.) Gansun Sci-tech Press: P. R. China, p. 20.

Clarke PJ, Davison EA and Fulloon L (2000) Germination and dormancy of grassy woodland and forest species: effects of smoke, heat, darkness and cold. *Australian Journal of Botany* 48: 687–699.

Clement SL, Griswold TL, Rust RW, Hellier BC and Stout DM (2006) Bee associates of flowering *Astragalus* and *Onobrychis* genebank accessions at a Snake River site in Eastern Washington. *Journal of the Kansas Entomological Society* 79: 254–260.

Cooper CS (1972) Establishment, hay yield, and persistence of two sainfoin growth types seeded alone and with low growing grasses and legumes. *Agronomy Journal* 64: 379–381.

Cox-Foster DL, Conlan S, Holmes EC, Palacios G, Evans JD, Moran NA, Quan P, Briese T, Hornig M, Geiser DM, Martinson V, vanEngelsdorp D, Kalkstein AL, Drysdale A, Hui J, Zhai J, Cui L, Hutchison SK, Simons JF, Egholm M, Pettis JS and Lipkin WI (2007) A metagenomic survey of microbes in honey bee colony collapse disorder. *Science* 318: 283–287.

Czochanska Z, Foo LY, Newman RH and Porter LJ (1980) Polymeric proanthocyanidins. Stereochemistry, structural units, and molecular weight. *Journal of the Chemical Society, Perkin Transactions* I: 2278–2286.

Dewick PM (1977) Biosynthesis of pterocarpan phtyoalexins in *Trifolium pratense*. *Phytochemistry* 16: 93–97.

Doyle CJ, Thomson DJ and Sheehy JE (1984) The future of sainfoin in british agriculture – an economic assessment. *Grass and Forage Science* 39: 43–51.

Dubbs AL (1968) The performance of sainfoin and sainfoin-grass mixture on dryland in central Montana. *Sainfoin symposium; 1968 December 12-13, Bozeman, MT: Montana Agricultural Experiment Station Bulletin* 627: 22–25.

Egan AR and Ulyatt MJ (1980) Quantitative digestion of fresh herbage by sheep. VI. Utilization of nitrogen in five herbages. *Journal of Agricultural Science* 94: 47–56.

Eischen FA, Graham RH and Rivera R (2009) Optimum time for feeding protein to wintering honey bee colonies in preparation for almond pollination. *Proceedings of the American Bee Research Conference*, 4–6 February, 2009, Gainesville, FL2009587.

Eken C, Demirci E and Dane E (2004) Species of *Fusarium* on sainfoin in Erzurum, Turkey. *New Zealand Journal of Agricultural Research* 47: 261–263.

Emre I, Turgut-balik D, Sahin A and Kursat M (2007) Total electrophoretic band patterns of some *Onobrychis* species growing in Turkey. *American-Eurasian Journal of Agricultural and Environmental Sciences* 2: 123–126.

FAO (2004) http://cnia.inta.gov.ar/helminto/Guidelines/collabo.pdf; http://cnia.inta.gov.ar/helminto/Guidelines/cap2.pdf

Frame J, Charlton JFL and Laidlaw AS (1998) *Temperate forage legumes*. CAB International.

Fraser MD, Fychan R and Jones R (2000) Voluntary intake, digestibility and nitrogen utilization by sheep fed ensiled forage legumes. *Grass Forage Science* 55: 271–279.

Gea A, Stringano E, Brown RH and Mueller-Harvey I (2011) *In situ* analysis and structural elucidation of sainfoin (*Onobrychis viciifolia*) tannins for high throughput germplasm screening. *Journal of Agricultural and Food Chemistry* (doi:http://pubs.acs.org/doi/abs/10.1021/jf103609p).

Givens DI and Rulquin H (2004) Utilisation by ruminants of nitrogen compounds in silage-based diets. *Animal Feed Science and Technology* 114: 1–18.

Givens DI and Shingfield KJ (2004) Foods derived from animals: the impact of animal nutrition on their nutritive value and ability to sustain long-term health. *Nutrition Bulletin* 29: 325–332.

Goplen BP, Richards KW and Moyer JR (1991) Sainfoin for Western Canada, Agriculture Canada Publication 1470/E.

Gray FA, Shigaki T, Koch DW, Delaney RD, Gray AM, Majerus ME, Cash D, Ditterline RL and Wichman DM (2006) Registration of 'Shoshone' Sainfoin. *Crop Science* 46: 988.

Grabber J, Broderick G, Russelle M, Powell M, Rotz A, Muck R, Mertens D and Massingill L (2002) Tannin-containing forage crops: a way to improve nitrogen use and profitability of dairy farms? http://www.dfrc.ars.usda.gov/cd/posters/Tannin%20dafosym.pdf

Guner A, Ozhatay N, Ekim T and Baser K (2000) Flora of Turkey and East Aegean Islands. In: Press EU (ed.) vol. 11, pp. 98–99.

Hanley ME, Franco M, Pichon S, Darvill B and Goulson D (2008) Breeding system, pollinator choice and variation in pollen quality in British herbaceous plants. *Functional Ecology* 22: 592–598.

Häring DA, Scharenberg A, Heckendorn F, Dohme F, Lüscher A, Maurer V, Suter D and Hertzberg H (2008) Tanniferous forage plants: agronomic performance, palatability and efficacy against parasitic nematodes in sheep. *Renewable Agriculture and Food Systems* 23: 19–29.

Harrison MJ (1998) Development of the arbuscular mycorrhizal symbiosis. *Current Opinion in Plant Biology* 1: 360–365.

Hart SP and Sahlu T (1993) Mohair production and body-weight gains of yearling Angora goats grazing forages with different tannin levels. *Proceedings of XVII International Grassland Congress,* Palmerston North, New Zealand. 575.

Hartlib S (1652) *His legacy*.

Hartwig UA and Nosberger J (1996) Symbiotic nitrogen fixation: a physiological link between plant and environment. In: Younie D (ed.) *Legumes in Sustainable Farming Systems*. Occasional Symposium no. 30. British Grassland Society. pp. 36–43.

Haynes RJ (1980) Competitive aspects of the grass-legume association 227-256. In: Brady NC (ed.) *Advances in Agronomy*. 33 New York: Academic Press. ISBN 0-12-000 733-39.

Hedqvist H, Mueller-Harvey I, Reed JD, Krueger CG and Murphy M (2000) Characterisation of tannins and *in vitro* protein digestibility of several *Lotus corniculatus* varieties. *Animal Feed Science and Technology* 87: 41–56.

Hendrikx P, Chauzat M-P, Debin M, Neuman P, Fries I, Ritter W, Brown M, Mutinelli F, Le Conte Y and Gregorc A (2009) Bee mortality and bee surveillance in Europe. Scientific report submitted to EFSA. CFP/EFSA/AMU/2008/02. Published 3 December 2009.

Hill R (1997) Sainfoin: the not quite forgotten legume. In: Lane GPF and Wilkinson JM (eds) *Alternative Forages for Ruminants*. Papers, Conference, Cirencester, pp. 55–59.

Horne M (1995) Pollen preference and its relationship to nesting success of *Megachile rotundata* (Hymenoptera, Megachilidae). *Annals of the Entomological Society of America* 88: 862–867.

Hoste H, Jackson F, Athanasiadou S, Thamsborg SM and Hoskin SO (2006) The effects of tannin-rich plants on parasitic nematodes in ruminants. *Trends in Parasitology* 22: 253–261.

Hoste H, Jackson F, Athanasiadou S, Thamsborg SM and Hoskin SO (2006) The effects of tannin-rich plants on parasitic nematodes in ruminants. *Trends in Parasitology* 22: 253–261.

Howes FN (2007) *'Plants and Beekeeping'*. London: Faber & Faber, p. 260.

Hughes SJ (1949) Studies on some diseases of sainfoin (*Onobrychis sativa*) II. The life history of *Ramularia onobrychidis* Allescher. *Transactions British Mycological Society* 32: 34–59.

Hutchinson JB (1965) *Crop Plant Evolution: A General Discussion*. *Essays on Crop Plant Evolution*. London: Cambridges University Press, pp. 166–181.

Ingham JL (1978) Flavonoid and isoflavonoid compounds from leaves of sainfoin (*Onobrychis viciifolia*). *Zeitschrift fuer Naturforschung* 33c: 146–148.

John A and Lancashire JA (1981) Aspects of the feeding and nutritive value of Lotus species. *Proceedings of the New Zealand Grasslands Association* 42: 152–159.

Jones GA, McAllister TA, Muir AD and Cheng KJ (1994) Effects of sainfoin (*Onobrychis viciifolia* Scop.) condensed tannins on growth and proteolysis by four strains of ruminal bacteria. *Applied and Environmental Microbiology* 60: 1374–1378.

Jones WT and Mangan JL (1977) Complexes of the condensed tannins of sainfoin (*Onobrychis viciifolia* Scop.) with Fraction 1 leaf protein and with submaxillary mucoprotein, and their reversal by polyethylene glycol and pH. *Journal of the Science of Food and Agriculture* 28: 126–136.

Jones WT, Broadhurst RB and Lyttleton JW (1976) The condensed tannins of pasture legume species. *Phytochemistry* 15: 1407–1409.

Karnezos TP, Matches AG and Brown CP (1994) Spring lamb production on alfalfa, sainfoin, and wheatgrass pastures. *Agronomy Journal* 86: 497–502.

Kells A (2001) Sainfoin: an alternative forage crop for bees. *Bee World* 82: 192–194.

Kidambi SP, Mahan JR and Matches AG (1990) Purification and thermal dependence of glutathione reductase from two forage legume species. *Plant Physiology* 92: 363–367.

Kingston-Smith AH, Bollard AL, Shaw RK, Davies TE and Theodorou MK (2003) Correlations between protein content and protease activity in forage crops. *Aspects of Applied Biology* 70: 101–105.

Koivisto JM and Lane GPF (2001) *Sainfoin Worth Anothe Look*. http://www.fao.org/AG/AGp/agpc/doc/Gbase/AddInfo/sainfoin.pdf

Koter Z (1965) Nutrition of Leguminous plants. 1. The effect of different forms of N on the growth and symbiotic N_2-fixation by red clover and sainfoin plants. 2. The effect of increasing amount of N on the yield and N_2-fixation by Sainfoin (*Onobrychis viciifolia* var. persica). *Pamielnik pulawski* 20: 3–37.

Koupai-Abyazani M, McCallum J and Bohm BA (1992) Identification of the constituent flavanoid units in sainfoin proanthocyanidins by reversed-phase high-performance liquid chromatography. *Journal of Chromatography* 594: 117–123.

Koupai-Abyazani M, Muir AD, Bohm BA, Towers GHN and Gruber MY (1993a) The proanthocyanidin polymers in some species of *Onobrychis*. *Phytochemistry* 34: 113–117.

Koupai-Abyazani M, McCallum J, Muir AD, Bohm BA, Towers GHN and Gruber MY (1993b) Developmental changes in the composition of proanthocyanidins from leaves of sainfoin (*Onobrychis viciifolia* Scop.) as determined by HPLC anlaysis. *Journal of Agricultural and Food Chemistry* 41: 1066–1070.

Lees GL, Suttill NH and Gruber MY (1993) Condensed tannins in sainfoin. 1. A histological and cytological survey of plant tissues. *Canadian Journal of Botany* 71: 1147–1152.

Li Y-G, Tanner G and Larkin P (1996) The DMACA-HCl protocol and the threshold proanthocyanidin content for bloat safety in forage legumes. *Journal of the Science of Food and Agriculture* 70: 89–101.

Liu Z (2006) Developing a modern agronomy for sainfoin (*Onobrychis viciifolia* Scop.). PhD Thesis, Coventry University in association with Royal Agricultural College.

Liu Z, Lane GPF and Davies WP (2006) The effects of establishment method on the yield of sainfoin (*Onobrychis viciifolia*) and sainfoin–grass mixtures. *Proceedings of British Grassland Society 8th Research Conference*.

Lu Y, Sun Y, Foo LY, McNabb WC and Molan AL (2000) Phenolic glycosides of forage legume *Onobrychis viciifolia*. *Phytochemistry* 55: 67–75.

MAAREC (2006) Mid-Atlantic Apiculture Research and Extension Consortium (MAAREC) – CCD Working Group. Colony Collapse Disorder Preliminary Report, p. 22.

Manning R (2001) Fatty acids in pollen: a review of their importance for honey bees. *Bee World* 82: 60–75.

Manning R (2006) Fatty acid composition of pollen and the effect of two dominant fatty acids (linoleic and oleic) in pollen and flour diets on longevity and nutritional composition of honey bees (*Apis mellifera*). PhD Thesis, Murdoch University.

Marais JPJ, Mueller-Harvey I, Brandt EV and Ferreira D (2000) Polyphenols, condensed tannins and other natural products in *Onobrychis viciifolia* (Sainfoin). *Journal of Agricultural and Food Chemistry* 48: 3440–3447.

Marten GC, Ehle FR and Ristau EA (1987) Performance and photosensitization of cattle related to forage quality of four legumes. *Crop Science* 27: 138–145.

Martiniello P (2005) Recurrent phenotypic selection of legumes and grass perennial forage crops using Mediterranean autochthonous germplasm. *Journal of Genetics and Breeding* 59: 3–4.

Mathre D (1968) Disease in sainfoin. *Sainfoin Symposium*: 65–66.

McAllister TA, Martinez T, Bae HD, Muir AD, Yanke LJ and Jones GA (2005) Characterization of condensed tannins purified from legume forages: chromophore production, protein precipitation, and inhibitory effects on cellulose digestion. *Journal of Chemical Ecology* 31: 2049–2067.

McGregor SE (1976) Insect pollination of cultivated crop plants, Chapter 4 in 'Legumes and Some Relatives'. Available at http://gears.tucson.ars.ag.gov/book/

McMahon LR, McAllister TA, Berg BP, Majak W, Acharya SN, Popp JD, Coulman BE, Wang Y and Chen K-J (2000) A review of the effects of forage condensed tannins on ruminal fermentation and bloat in grazing cattle. *Canadian Journal of Plant Science* 80: 469–485.

Meyer DW (1975) Yield, regrowth and persistence of sainfoin under fertilisation. *Agronomy Journal* 67: 439–441.

Meyer DW and Badaruddin M (2001) Frost tolerance of ten seedling legume species at four growth stages. *Crop Science* 41: 1838–1842.

Min BR, Barry TN, Attwood GT and McNabb WC (2003) The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. *Animal Feed Science and Technology* 106: 3–19.

Min BR, McNabb WC, Barry TN and Peters JS (2000) Solubilization and degradation of ribulose-1,5-*bis*phosphate carboxylase/oxygenase (*EC* 4.1.1.39; Rubisco) protein from white clover (*Trifolium repens*) and *Lotus corniculatus* by rumen microorganisms and the effect of condensed tannins on these processes. *Journal of Agricultural Science (Cambridge)* 134: 305–317.

Molle G, Decandia M, Soelter U, Greef JM, Rochon JJ, Sitzia M, Hopkins An and Rook AJ (2008) The effect of different legume-based swards on intake and performance of pgrazing ruminants under Mediterranean and cool temperate conditions. *Grass and Forage Science* 63: 513–530.

Morrill WL, Ditterline RL and Cash SD (1998) Insect pests and associated root pathogens of sainfoin in western USA. *Field Crops Research* 59: 129–134.

Moyer JR (1985) Effect of weed control and a companion crop on alfalfa and sainfoin establishment, yields and nutrient composition. *Canada Journal of Plant Science* 65: 107–116.

Mueller-Harvey I (2006) Unravelling the conundrum of tannins in animal nutrition and health. *Journal of the Science of Food and Agriculture* 86: 2010–2037.

Mueller-Harvey I (2009) 'Holy hay' – re-inventing a traditional animal feed. *Biologist* 56: 22–27.

Mueller-Harvey I and Dhanoa MS (1991) Varietal differences among sorghum crop residues in relation to their phenolic HPLC fingerprints and responses to different environments. *Journal of the Science of Food and Agriculture* 57: 199–216.

Negri V and Cenci CA (1988) Morphological characterization of natural populations of *Onobrychis viciifolia* (Leguminosae) from Central Italy. *Willdenowia* 17: 19–31.

Negri V, Veronesi F and Falcinelli M (1987) Germplasm evaluation of *Onobrychis viciifolia* Scop. for agronomic traits. *Genetica Agraria*.

Newman G (1997) An overview of forage legumes. In: GPF Lane and JM Wilkinson (eds) *Alternative Forages for Ruminants*. Cirencester, UK, pp. 3–14.

Ojeda-Robertos N, Manolaraki F, Theodoridou K, Aufrere J, Halbwirth H, Stich K, Regos I, Treutter D, Mueller-Harvey I and Hoste H (2010) The anthelmintic effect of sainfoin (silage, hay, fresh) and the role of flavonoid glycosides. *61st Annual Meeting, European Association of Animal Production*, Heraklion, Crete, Greece. 23–27 August 2010.

Paolini V, De La Farge F, Prevot F, Dorchies PH and Hoste H (2005) Effects of the repeated distribution of sainfoin hay on the resistance and the resilience of goats naturally infected with gastrointestinal nematodes. *Veterinary Parasitology* 127: 277–283.

Parker RJ and Moss BR (1981) Nutritional value of sainfoin hay compared with alfalfa hay. *Journal of Dairy Science* 64: 206–210.

Pecetti L, Annicchiarico P, Battini F and Cappelli S (2009) Adaptation of forage legume species and cultivars under grazing in two extensive livestock systems in Italy. *European Journal of Agronomy* 30: 199–204.

Piper CV (1924) *Forage Plants and their Culture*. New York: The Macmillan Co.

Potts SG, Roberts SPM, Dean R, Marris G, Brown M, Jones R and Settele J (2010) Declines of managed honeybees and beekeepers in Europe. *Journal of Apicultural Research* 49: 15–22.

Prévost D, Bordeleau LM and Antoun H (1987) Symbiotic effectiveness of indigenous arctic rhizobia on temperate forage legume – sainfoin (*Onobrychis viciifolia*). *Plant and Soil* 104: 63–69.

Provorov NA and Tikhonovich IA (2003) Genetic resources for improving nitrogen fixation in legume-rhizobia symbiosis. *Genetic resources and Crop Evoloutuion* 50: 89–99.

Regos I, Urbanella A and Treutter D (2009) Idenficiation and quantification of phenolic compounds from the forage legume sainfoin (*Onobrychis viciifolia*). *Journal of Agricultural Food and Chemistry* 57: 5843–5852.

Rios-de Álvarez L, Greer AW, Jackson F, Athanadiadou S, Kyriazakis I and Huntley JF (2008) The effect of dietary sinafoin (*Onobrychis viciifolia*) on local cellular responses to *Trichostrongylus colubriformis* in sheep. *Parasitology* 135: 117–1124.

Rochon JJ, Doyle CJ, Greef JM, Hopkins A, Molle G, Sitzia M, Scholefield D and Smith CJ (2004) Grazing legumes in Europe: a review of their status, management, benefits, research needs and future prospects. *Grass and Forage Science* 59: 197–214.

Rosov SA (1952) Sainfoin - one of the best honey plants in crop rotation. *Pchelovodstvo Mosk* 29: 46–52.

Rozen JG, Ozebek H, Ascher JS, Sedivy C, Praz C, Monfared A and Mueller A (2010) Nests, petal usages, floral preferences, and immatures of *Osmia (Ozbekosmia) avosetta* (Megachilidae: Megachilinae: Osmiini), including biological comparisons with other Osmiine bees. *American Museum Novitates* 3680: 1–22.

Rumball W and Claydon RB (2005) Germplasm release – 'G35' Sainfoin (*Onobrychis viciifolia* Scop.). *New Zealand Journal of Agricultural Research* 48: 127–128.

Russell GB, Shaw GJ, Christmas PE, Yates MB and Sutherland RW (1984) Two 2-arylbenzofurans as insect feeding deterrents from sainfoin (*Onobrychis viciifolia*). *Phytochemistry* 23: 1417–1420.

Sánchez-Yélamo MD (2006) Rapid characterisation of some species of the genus *Onobrychis* Miller (Leguminosae: Papilionoideae) by isozyme methods. *Seed Science and Technology* 34: 297–306.

Scharenberg A, Arrigo Y, Gutzwiller A, Wyss U, Hess HD, Kreuzer M and Dohme F (2007) Effect of feeding dehydrated and ensiled tanniferous sainfoin (*Onobrychis viciifolia*) on nitrogen and mineral digestion and metabolism of lambs. *Archives of Animal Nutrition* 61: 390–405.

Scharenberg A, Heckendorn F, Arrigo Y, Hertzberg H, Gutzwiller A, Hess HD, Kreuzer M and Dohme F (2008) Nitrogen and mineral balance of lambs artificially infected with *Haemonchus contortus* and fed tanniferous sainfoin (*Onobrychis viciifolia*). *Journal of Animal Science* 86: 1879–1890.

Shan MH, Singh KN, Kachroo D and Khanday BA (1991) Performance of lucerne and sainfoin under different cuttings and levels of phosphorus. *Indian Journal of Agronomy* 36: 61–66.

Shaw AL (1968) The certification program for sainfoin in Montana. *Sainfoin Symposium Montana. Agricultural Experiment Station Bulletin*: 77–78.

Sheehy JE, Minchin FR and McNeill A (1984) Physiological principles governing the growth and development of lucerne, sainfoin and red clover. In *Forage Legumes*. Proceedings of a Symposium, February 21–23, 1984. British Grassland Society, pp. 113–125.

Sheehy JE and Popple SC (1981) Photosynthesis, water relations, temperature and canopy structure as factors influencing the growth of sainfoin (*Onobrychis viciifolia* Scop.) and Lucerne (*Medicago sativa* L.). *Annals of Botany* 48: 113–128.

Sheldrick R, Thomson D and Newman G (1987) Sainfoin. In: *Legumes for Milk and Meat*. Chalcombe Publications: Marlow, UK, pp. 59–69.

Sheldrick RD and Thomson DJ (1982) Management and Utilization of Sainfoin (*Onobrychis Sativa*) Information Leaflet no. 13.

Sims JR, Muir MK and Carleton AE (1968) Evidence of ineffective rhizobia and its relation to the nitrogen nutrition of sainfoin (*Onobrychis viciaefolia*). *Sainfoin Symposium Montana. Agricultural Experiment Station Bulletin* 627: 8–12.

Širjaev G (1925) *Onobrychis generis revisio critica*. Brno.

Sprent J (2003) Plant biology: Mutual sanctions. *Nature* 422: 672–674.

Stewart JL, Mould F and Mueller-Harvey I (2000) The effect of drying treatment on the fodder quality and tannin content of two provenances of *Calliandra calothyrsus* Meissner. *Journal of the Science of Food and Agriculture* 80: 1461–1468.

Stringano E, Hayot C, Smith L, Theodoridou K, Aufrére J, Brown RH, Hayes W, Cramer R and Mueller-Harvey I (2010) Thiolytic screening method for exploring condensed tannin variation in a unique sainfoin germplasm bank. 58th International Congress and Annual Meeting of the Society of Medicinal Plant and Natural Product Research and 7th Tannin Conference, 29th Aug- 2th Sep 2010. Berlin, Germany. *Planta Medica* 76: 1166.

Syngenta (2008) Ensuring there is honey still for tea. http://www.syngenta.com/en/features/honey_tea.htm

Taki H, Okabe K, Makino S, Yamaura Y and Sueyoshi M (2009) Contribution of small insects to pollination of common buckwheat, a distylous crop. *Annals of Applied Biology* 155: 121–129.

Tamas E (2006) Citological aspects of the *Onobrychis* genus. Bulletin of University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca. *Agriculture* 62: 154–158.

Tamminga S (1992) Nutrition management of dairy cows as a contribution to pollution control. *Journal of Dairy Science* 75: 345–357.

Tamminga S, Bannink A, Kijkstra J and Zom R (2007) Feeding strategies to reduce methane loss in cattle. Report 34. Animal Sciences Group, Wageningen University, The Netherlands. p. 44 http://www2.asg.wur.nl/NR/rdonlyres/F81D8745-6596-4296-A292-8553950E2B98/42707/34.pdf (checked on 20 April 2010).

Tasei JN (1984) Forage and protein legumes. In: Pesson PL (ed.) *Pollination and Plant Productions*. Paris: INRA, pp. 261–308.

Tasei JN and Aupinel P (2008) Nutritive value of 15 single pollens and pollen mixes tested on larvae produced by bumblebee workers (*Bombus terrestris,* Hymenoptera: Apidae). *Apidologie* 39: 397–409.

Theodoridou K, Aufrère J, Andueza D, Pourrat J, Le Morvan A, Stringano E, Mueller-Harvey I and Baumont R (2010) Effects of condensed tannins in fresh sainfoin (*Onobrychis viciifolia*) on *in vivo* and *in situ* digestion in sheep. *Animal Feed Science and Technology* 160: 23–38.

Thomson DJ (1982) Nutritive and utilisation value of sainfoin. In: *The Future of Sainfoin in British Agriculture*. Hurley: The Grassland Research Institute, pp. 8–11.

Thomson DJ, Beever DE, Harrison DG, Hill IW and Osbourn DF (1971) The digestion of dried lucerne (*Medicago sativa*, L.) and dried sainfoin (*Onobrychis viciifolia*, Scop.) by sheep. *Proceedings of the Nutrition Society* 30: 14A.

Thomson JR (1951a) Sainfoin in its first harvest year. *Journal of British Grassland Society* 6: 107–117.

Thomson JR (1951b) Seed studies in sainfoin. *Journal of British Grassland Society* 6: 147–159.

Thomson JR (1952) Further seed studies in sainfoin. *Grass and Forage Science* 7: 65–69.

Tufenkci S, Erman M and Sonmez F (2006) Effects of phosphorus and nitrogen applications and Rhizobium inoculation on the yield and nutrient uptake of sainfoin (*Onobrychis viciifolia* L.) under irrigated conditions in Turkey. *New Zealand Journal of Agricultural Research* 49: 101–105.

USDA SARE (2007) *Managing Cover Crops Profitably*, 3rd edn. http://www.sare.org/publications/covercrops/buckwheat.shtml, p. 244.

Van Engelsdorp D, Underwood R, Caron D and Hayes J Jr (2007) An estimate of managed colony losses in the winter of 2006–2007: a report commissioned by the apiary inspectors of America. *American Bee Journal* 147: 599–603.

Waghorn G (2008) Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat

production – progress and challenges. *Animal Feed Science and Technology* 147: 116–139.

Waghorn GC, Jones WT, Shelton ID and McNabb WC (1990) Condensed tannins and the nutritive value of herbage. *Proceedings of the New Zealand Grasslands Association* 51: 171–176.

Waghorn GC and McNabb WC (2003) Consequences of plant phenolic compounds for productivity and health of ruminants. *Proceedings of the Nutrition Society* 62: 383–392.

Wallace LE (1968) Current and potential insect problems of sainfoin in America. *Sainfoin Symposium Montana. Agricultural Experiment Station Bulletin* 627: 67–70.

Waller PJ (2006) From discovery to development: current industry perspectives for the development of novel methods of helminth control in livestock. *Veterinary Parasitology* 139: 1–14.

Wang Y, Barbieri LR, Berg BP and McAllister TA (2007) Effects of mixing sainfoin with alfalfa on ensiling, ruminal fermentation and total tract digestion of silage. *Animal Feed Science and Technology* 135: 296–314.

Wang Y, Berg BP, Barbieri LR, Veira DM and McAllister TA (2006) Comparison of alfalfa and mixed alfalfa-sainfoin pastures for grazing cattle: effects on incidence of bloat, ruminal fermentation, and feed intake. *Canadian Journal of Animal Science* 86: 383–392.

Westphal C, Bommarco R, Lamborn E, Petanidou T, Potts SG, Roberts SPM, Szentgyörgyi H, Vaissière BE, Woyciechowski M and Steffan-Dewenter I (2008) Measuring bee biodiversity in different habitats and biogeographic regions. *Ecological Monographs* 78: 653–671.

Wiesner LE, Carleton AE and Cooper CS (1968) Factors affecting sainfoin seed germination and emergence. *Sainfoin Symposium Montana. Agricultural Experiment Station Bulletin*: 13–15.

Williams PH and Osborne JL (2009) Bumblebee vulnerability and conservation world-wide. *Apidologie* 40: 367–387.

Yildiz B, Ciplak B and Aktoklu E (1999) Fruit morphology of sections of the genus *Onobrychis* Miller (Fabaceae) and its phylogenetic implications. *Israel Journal of Plant Sciences* 47: 269–282.