Promising options for improving performance and proanthocyanidins of the forage legume sainfoin (*Onobrychis viciifolia* Scop)

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**Promising options for improving performance and proanthocyanidins
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32 **Abstract**

33 Sainfoin (*Onobrychis viciifolia* Scop.) is an underutilized perennial forage legume with high
34 potential as a forage source for ruminants in grassland based livestock production systems. The
35 species is also particularly valued for its content of proanthocyanidins (PA; also known as
36 condensed tannins), which have been shown to improve animal health by reducing bloat, and by
37 diminishing gastro-intestinal parasites, can reduce nitrogen losses through excreted urine and may
38 also have the potential to lower methane emissions. However, sainfoin cultivation is not widespread
39 today, mainly due to the limited availability of high performing cultivars and agronomic constraints
40 such as slow establishment, poor competitive ability and limited yield stability. In this paper, we
41 give an overview on the importance and the potential of sainfoin and review recent findings
42 regarding cultivation practices and the variability observed for agronomic and quality traits. A
43 special focus is placed on the potential and implications for targeted improvement through breeding.
44 Results show that stability of sainfoin yields can be significantly improved when grown in mixtures
45 with appropriate companion species and it was shown that the choice of cultivar, management
46 practices and drought could have an impact on PA content and composition. Various studies
47 demonstrate large variability in agronomic performance, PA concentration and PA composition
48 among and within sainfoin accessions, highlighting the big potential to improve this species by
49 breeding. In addition, we highlight recent advances in breeding research such as high rates of self-
50 fertilization in this generally allogamous species and the development of molecular genetic
51 resources, which build the basis for novel breeding strategies and the targeted exploitation of
52 sainfoin germplasm in the future.

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59 **Keywords:**

60 Forage legume, condensed tannins, cultivation practices, marker assisted breeding, self-fertilization

61

62 **Introduction**

63 Temporary and permanent grassland provide the backbone for sustainable livestock production in
64 temperate regions worldwide. They not only cover 37 percent of the earth's surface and significantly
65 contribute to food security, but also have an invaluable potential to store and further sequesterate
66 carbon, regulate water regimes and serve as an important reservoir of biodiversity for both, plant
67 species and pollinating insects (Carlier et al. 2009; O'Mara 2012). In Europe, centuries of grassland
68 farming led to the establishment of distinct plant communities in natural, semi-natural and
69 agriculturally improved grassland, which form an important source of forage for ruminants used in
70 milk and meat production. Even more importantly, a range of forage grass and legume species such
71 as ryegrasses (*Lolium* spp.), fescues (*Festuca* spp.), alfalfa (*Medicago sativa*) and clovers (*Trifolium*
72 spp.), are sown as forage crops in crop rotations. While monocultures of highly productive grass
73 species produce high forage yields (Humphreys et al. 2010), they require high nitrogen fertilizer input
74 and the forage produced often does not meet the concentrations of crude protein and minerals required
75 for a balanced ruminant diet. Forage legumes on the other hand are particularly valued for their high
76 protein content and their ability to fix atmospheric nitrogen (Frame 2005), but they often cannot
77 provide the energy needed for reaching the full productivity potential of modern livestock breeds.
78 The use of mixed legume – grass swards for forage production not only results in a more balanced
79 composition of the forage, it also substantially reduces the nitrogen fertilizer needs and leads to
80 substantially higher forage yield and higher animal intake (reviewed in Lüscher et al. 2014). In
81 addition, some forage legume species contain specific features such as tannins, polyphenol oxidases
82 or protease enzymes, which may benefit ruminant nutrition, increase animal health and have the
83 potential to reduce greenhouse gas emissions (Kingston-Smith et al. 2010; Mueller-Harvey 2006).
84 Despite a long history of cultivation of a relatively broad range of forage legume species including
85 medics (*Medicago* spp.), clovers (*Trifolium* spp.) and vetches (*Vicia* spp.), today's grassland
86 agriculture largely depends on three main legume species, alfalfa, red clover (*T. pratense* L.) and
87 white clover (*T. repens* L.). These are either mainly grown in monoculture (alfalfa) or mixtures
88 (clovers; Annicchiarico et al. 2015). Consequently, breeding efforts and achievements focused on
89 these species (Abberton and Marshall 2010; Boller et al. 2010b; Veronesi et al. 2010), while minor
90 species such as birdsfoot trefoil (*Lotus corniculatus* L.) and sainfoin (*Onobrychis viciifolia* Scop.)
91 received only little attention from plant breeders and plant scientists over the past decades (Piano and
92 Pecetti 2010). However, some of these minor legume species may be particularly adapted to specific
93 environments based on their physiological and morphological properties or particularly valuable for
94 animal nutrition due to their content in plant secondary metabolites. Sainfoin may for example be
95 particularly suited for alkaline and drought prone soils, which cover large proportions of Central and
96 Southern Europe (Sölter et al. 2007) and are likely to increase in abundance due to climate change

97 (Trnka et al. 2011). In addition, promising concentrations of proanthocyanidins (PA; also referred to
98 as condensed tannins) have been found in different sainfoin accessions and cultivars (Azuhnwí et al.
99 2011; Häring et al. 2007; Stringano et al. 2012), which, fed in sufficient concentrations, can
100 significantly improve animal health by reducing bloat and by diminishing gastro-intestinal parasites
101 (Azuhnwí et al. 2013b; Wang et al. 2006).

102 Despite these apparent advantages, sainfoin cultivation is not widespread today, mainly due to the
103 limited availability of high performing cultivars and agronomic constraints such as slow
104 establishment, limited competitive ability and yield stability, as well as sensitivity to water logging
105 (Häring et al. 2008; Heinrichs 1970; Koch et al. 1972). In this paper, we give an overview on the
106 importance and the potential of the underutilized forage legume sainfoin and review recent advances
107 in the area of (i) agronomic practices for constantly high sainfoin yield of good quality (ii) the
108 variability of PA content and composition within and among accessions and its stability over different
109 environments (lack of GxE interactions), building the basis for the selection of improved cultivars
110 and (iii) recent advances in the area of sainfoin genetics and the development of molecular genetic
111 tools. All three together will provide the basis for developing optimized breeding strategies for this
112 orphan forage legume.

113

114 **Sainfoin – a promising forage legume**

115 Sainfoin, *Onobrychis viciifolia* Scop, is a perennial herbaceous forage legume, grown as a forage
116 source for ruminants in grassland based livestock production systems. Its biomass is composed of
117 rather rigid stems and multifoliate leaflets with up to 30 pinnate and one terminal leaflet. Sainfoin is
118 usually cut and used fresh or preserved as hay or pellets, while grazing is restricted to dry areas with
119 moderate irrigation (Wilman and Asiedu 1983). In addition, a large potential for silage production
120 has also been reported (Copani et al. 2016; Grosse Brinkhaus et al. 2016). Dry matter yields range
121 between 2 and 12 t ha⁻¹ and are therefore considerably lower when compared to alfalfa or red clover
122 (Table 1; Frame et al. 1998). However, forage quality remains high even when cut in full flowering
123 stage due to the fact that the leaves are less prone to fall off when compared to alfalfa (Goplen et al.
124 1991). The species is also particularly valued for its PA content, contributing to animal health and
125 reducing the ecological fingerprint of livestock production as will be discussed in more detail below.
126 Sainfoin is also able to withstand drought spells and to thrive in soils with limited water availability
127 thanks to a long taproot which can reach depths of up to two meters in the soil (Fleischmann 1932).
128 In addition to a strong taproot, sainfoin is characterised by an immense fine root system which can
129 contribute up to 16,200 kg of dry matter ha⁻¹ (four times that of alfalfa), thus significantly improving
130 soil fertility (Sergeeva 1955).

131 Sainfoin belongs to the family Fabaceae, subfamily Faboidae and the tribe Hedysarae. The tribe
132 Hedysarae has a rather controversial taxonomy, but in general it has been accepted for sainfoin to
133 belong to the genus *Onobrychis* of the section *Onobrychis* (Woodgate et al. 1999). However, sainfoin
134 still is known under a range of different synonyms such as *Hedysarum onobrychis* L., *O. onobrychis*
135 (L.) Karsten, *O. sativa* Lam., *O. viciifolia* Scop. subsp. *sativa* (Lam.) Thell. and *O. vulgaris* Güldenst.
136 (Porcher 2004). The species is further separated into two botanical types: *O. viciifolia* Scop. var.
137 *communis*, the common type, has its origin in Central Europe and is also referred to as the single cut
138 type due to its slow regrowth after the first cut (Badoux 1964), while *O. viciifolia* Scop. var. *bifera*
139 Hort., the giant type, with origin in the Middle East is characterised by a faster regrowth after cut and
140 is therefore able to flower two or three times per year (Piano and Pecetti 2010). The species is
141 tetraploid with $2n = 4x = 28$ chromosomes. Based on cytological studies and preponderance of
142 tetrasomic gene segregation, autopolyploid inheritance was suggested, but indications for
143 allopolyploid inheritance have also been observed and no conclusive evidence was given so far (De
144 Vicente and Arus 1996; Sacristan 1966).

145 Sainfoin cultivation has a long history and dates back to the 10th century, when it is assumed to have
146 started in today's Armenia (Akopian 2009). From there, sainfoin spread across Europe, where, during
147 the 18th century, it became an important source of roughage for ruminants in countries such as France,
148 Italy, UK, Germany and Switzerland and was also introduced into the USA (Piper 1914). However,
149 with the increased availability of seed from high yielding forage legume species such as red clover
150 and alfalfa, the importance of sainfoin continuously decreased and, despite its numerous advantages,
151 the species is not widespread in today's intensive agriculture with high fertilizer inputs and cutting
152 frequencies. Consequently, breeding activities have remained on a very low level with only a few
153 listed sainfoin cultivars available (European Commission 2015). The main reasons for the poor
154 representation of sainfoin in productive grassland systems are its lower yield when compared to other
155 forage legumes, its poor competitive ability in early establishment, a limited persistence,
156 susceptibility to waterlogging (Liu et al. 2010; Sheehy and Popple 1981), low tolerance to frequent
157 cutting (Malisch et al. 2017) and susceptibility to diseases such as powdery mildew or phytophthora
158 root rot (Sears et al. 1975).

159

160 **Agronomic practices for high sainfoin yield of good quality**

161 *Improved mixtures for more stable sainfoin yields*

162 One of the main issues with sainfoin cultivation is its low competitive ability, which makes it
163 particularly vulnerable to competition by weeds or, when grown in mixtures, by partner species
164 (Häring et al. 2008). While weed control through herbicides is cost-intensive and may have undesired
165 environmental effects, grass-legume mixtures offer the possibility to significantly decrease the

166 invasion of unsown species in mixed stands (Connolly et al. 2017; Sanderson et al. 2012). However,
 167 in sainfoin some partner species were found not only to considerably suppress weed infestations, but
 168 also lead to a drastically reduced sainfoin yield when compared to monoculture (Häring et al. 2008).
 169 This may have been due to an inappropriate choice of the partner species or an unfavourable sowing
 170 density, which would allow the partner to establish too quickly. In a recent study with three sainfoin
 171 cultivars and six companion species, sainfoin-grass mixtures showed considerably lower shares of
 172 unsown species (2 to 4%) when compared to sainfoin monocultures (15 %; Malisch et al. 2017).
 173 Although yield patterns in the different mixtures / monocultures varied to some extent over the years,
 174 on average mixtures showed an annual total yield of 16.5 t ha⁻¹, which was higher by 33% than the
 175 mean yield of partner species in monoculture and by 21% when compared to sainfoin monoculture.
 176 Comparable effects such as transgressive overyielding are a feature of multispecies grass-legume
 177 mixtures where partner species optimally complement each other and N fertilizer input is moderate
 178 (reviewed in Lüscher et al. 2014).
 179 Although it is unclear which proportions of sainfoin are needed to optimally profit from its benefits,
 180 Wang et al. (2006) found that 35% of sainfoin in mixtures is sufficient to reduce bloat in grazing
 181 steers by 77%. In general, a legume proportion of 30 to 50% was identified to be ideal in terms of
 182 biomass and protein yield and forage quality (Lüscher et al. 2014; Suter et al. 2015). In this respect,
 183 mixtures with meadow fescue (*F. pratensis*) and perennial ryegrass (*L. perenne*) appear particularly
 184 valuable, as they resulted in consistently high sainfoin proportions of 38 to 63% (Malisch et al. 2017).
 185 The choice of appropriate cultivars may also substantially influence sainfoin yield, as the cultivar
 186 Perly achieved higher sainfoin yields and suppressed weeds better, in both, monocultures and
 187 mixtures than did the two other cultivars (Malisch et al. 2017). This result stresses the importance of
 188 cultivars with increased performance, especially with increased competitive ability (against weeds
 189 and the mixture partner). Strategies to achieve this include breeding for high yields in general
 190 (independent of monoculture or mixture) and breeding for high performance derived from the
 191 adaptation to specific mixture partners (Hill 1990; Lüscher et al. 1992; Lüscher and Jacquard 1991;
 192 Zuppinger-Dingley et al. 2014). Indeed, Sottie et al. (2014) report the development of a sainfoin
 193 population especially adapted for cultivation in mixtures with alfalfa.

194

195 *Symbiotic N₂ fixation and drought tolerance*

196 In addition to its benefit to animal health through its content of PAs and other polyphenolic
 197 compounds, high sainfoin proportions in the sward may also substantially contribute to sustainable
 198 livestock production through symbiotic nitrogen fixation. It was often argued that insufficient N₂
 199 fixation could be one factor contributing to the poor performance of sainfoin (Mora-Ortiz and Smith
 200 2016). However, recent results demonstrate, that sainfoin can fix up to 168 kg N ha⁻¹ via symbiosis,

201 with the proportions of apparent N derived from the atmosphere ranging from 70% (monoculture) to
202 84% (in mixture with perennial ryegrass), regardless whether seed was previously inoculated with
203 rhizobia or not (Malisch et al. 2017). This range was comparable to other legume species under
204 comparable growth conditions (Nyfeler et al. 2011; Oberson et al. 2013).
205 Sainfoin is able to withstand drought stress thanks to its large taproot. Although biomass reductions
206 of up to 37% have been observed in sainfoin after 14 weeks of drought, plants were able to persist
207 and regrow even under very low water availability (Malisch et al. 2016). In addition, considerable
208 drought tolerance has been observed in sainfoin ecotypes, which provides the basis for further
209 improvement of this important trait (Irani et al. 2015).

210

211 *Concentration and structural composition of PAs*

212 PAs are polymers of flavan-3-ol subunits and are commonly found in most woody plants such as
213 grapevine (*Vitis vinifera*), tea (*Camellia sinensis*) or pome fruits and are also present in several legume
214 species (Dixon et al. 2005). Sainfoin has been shown to contain significantly higher amounts of PAs
215 when compared to other forage legume species such as white clover or alfalfa (Aufrere et al. 2013;
216 Azuhnwili et al. 2013a) or to be higher yielding and more competitive compared to other PA containing
217 legumes such as big trefoil (*L. pedunculatus*) or birdsfoot trefoil (Häring et al. 2008; Häring et al.
218 2007). PAs are a highly diverse group of tannins, which can be further characterised by the proportion
219 of their flavanol units belonging to procyanidins (PC, i.e. catechin or epicatechin) or prodelphinidins
220 (PD, i.e. galliccatechin or epigallocatechin) and the length of the oligomer or polymer chains. Many
221 different PA structures including very long polymers exceeding 30 kDa have been identified in plant
222 species (Bravo 1998). In ruminants, PAs in the diet are associated with a broad range of benefits,
223 some of which are well understood while other need further research to fully exploit their potential
224 (Lüscher et al. 2014; Mueller-Harvey 2006). For example, PAs have the potential to precipitate
225 proteins by means of non-covalent hydrogen bonds between the phenolic groups of the PA and the
226 amide groups of the protein. In ruminants fed with protein rich diets, protein use efficiency is often
227 low due to very rapid protein degradation in the rumen which inhibits the complete utilization of
228 amino acids by rumen microbes (Broderick and Albrecht 1997). If PAs are present, proteins are
229 precipitated, which increases the fraction of rumen escape proteins and leads to lower protein
230 digestibility in the rumen. This not only leads to reduced N losses through excreted urine, it may also
231 lead to faster animal growth rates and increased milk production (Girard et al. 2016; Waghorn 2008).
232 Protein precipitation in the rumen also reduces fermentation gas accumulation and the formation of
233 stable foams, which both lead to bloat and may cause animal death (Sottie 2014; Wang et al. 2012).
234 PAs have also been shown to play a role in the control of gastrointestinal parasites by reducing the
235 nematode fecundity and by improving the animal's immune response (Hoste et al. 2012). Other

benefits of PAs in the diet include potential reduction of methane emissions (Hatew et al. 2015) and increased quality of animal products for human consumption (Girard et al. 2016). The PA concentrations in a forage, as well as their composition influence the positive effects PAs can have on ruminants. PAs in plants are constructed of either PC or PD subunits or of oligomers and polymers containing both, PC and PD subunits. This composition substantially influences bioactive properties of PAs. For example, susceptibility of parasites has been shown to strongly depend on PA structure, with PD-rich PAs generally showing higher anthelmintic properties when compared to PC-rich PAs (Kommuru et al. 2014; Quijada et al. 2015). However, PDs might result in decreased PA-protein dissociation in the abomasum and thus, in decreased nitrogen use efficiency (Mueller-Harvey 2006). In order for sainfoin to be widely used as a health promoting forage for ruminants, and to improve these traits through targeted breeding, detailed information on PA content and composition in cultivars and individual plants is needed.

248

249 **High variability fosters opportunities for breeding**

250 *High variability in concentration and composition of PAs*

251 Although several studies have demonstrated positive effects of sainfoin on animal health and
252 nutrition, due to analytical limitations, these studies largely focused on analysing total PA content
253 rather than PA composition, relying on methods such as thiolysis or HCl-butanol extraction (e.g.
254 Azuhnwi et al. 2013a; Azuhnwi et al. 2011). Recently, a novel method for UPLC-MS/MS analysis
255 has been developed which allows efficient characterisation of PA composition for characteristics such
256 as the mean degree of polymerization (mDP) or PC/PD ratios on a large number of individual plants
257 (Engström et al. 2014). This method was used to investigate PA content and composition in 27
258 sainfoin accessions and large variation in average PA concentration in leaves was found across all
259 accessions ranging from 23.0 mg g⁻¹ DM to 47.5 mg g⁻¹ DM (Table 1; Malisch et al. 2015).
260 Interestingly, average leaf PA concentrations were higher for ecotype populations when compared to
261 cultivars and landraces. This may have been due to the fact that breeding of sainfoin largely focused
262 on yield and persistence (Goplen et al. 1991) and shows the potential of untapped germplasm for
263 increasing PA in sainfoin. As expected for an outbreeding species, large variability in PA content and
264 composition was also observed between individual plants within accessions. PA content and
265 composition is also known to vary considerable within the plant, with young leaves having highest
266 and stems having lowest concentrations (Regos et al. 2009). In addition, sainfoin leaves appeared to
267 better meet the PA composition required for optimal health and nutritional benefits. They had longer
268 polymers and higher PD proportions when compared to stems and were also found to have higher
269 concentrations of rutin (quercetin-*O*-rutinoside) and nicotiflorin (kaempferol-*O*-rutinoside; Malisch

et al. 2015), both these components have been shown to enhance anthelmintic properties of PAs (Barrau et al. 2005).

Increasing PA yield

In order to improve sainfoin as a bioactive forage, not only PA concentration and composition, but also PA yield *per se* needs to be improved. Potentially, increasing biomass yield could restrict the increase of PA concentration through dilution, based on the fact that plant resources are limited and can only be allocated to either growth or the production of plant secondary metabolites (Stamp 2003). Indeed, a certain trade-off between plant yield and leaf PA concentration was observed for sainfoin, but this trade-off was comparatively small and was restricted to very small plants, which were in a yield range not relevant to plant breeders. As a consequence, the amount of PAs harvested per plant was significantly higher for cultivars (332 mg plant⁻¹) when compared to ecotype accessions (256 mg plant⁻¹; Malisch et al. 2015). In addition, no trade-off was found for PA composition and plant yield. Therefore, further yield gains could be possible without compromising PA content and composition in sainfoin (Malisch et al. 2015). Thus, the variability in sainfoin germplasm together with the analytical tools available, provide the basis for effective selection of sainfoin genotypes with an ameliorated PA content and composition.

Influence of growth stages and environment on PA properties

Another issue when growing sainfoin as a bioactive forage is the stability of PA content and composition across ontogenetic stages of the plant, growing seasons and different environments. The latter may be particularly important with regard to increasing occurrences of drought periods and rising temperatures through the impact of climate change (Trnka et al. 2011). In a large-scale experiment where artificial drought was imposed through rain-out shelters, PA concentration and composition were significantly affected by drought, but also clearly differed between plants in the vegetative and flowering stage, respectively (Malisch et al. 2016). Vegetative plants under drought showed significantly higher PA concentrations in their leaves when compared to rainfed control plants, but also when compared to plants in the reproductive stage exposed to drought. However, the simultaneous analysis of dry matter yield allowed to demonstrate, that increased PA concentrations under drought are presumably rather due to reduced biomass yield than to increased PA synthesis (Malisch et al. 2016).

PA content of sainfoin has been shown to be highly variable among cultivars and accessions (Azuhwi et al. 2013a; Malisch et al. 2015; Stringano et al. 2012). Averaged over years, different environments and treatments, these differences may be as high as 45% between the accessions with the highest and the lowest PA concentration, respectively. However, all accessions responded

305 similarly to drought with respect to the PA concentration and composition and there was no accession
306 x drought interaction observed (Malisch et al. 2016). Thus, the ranking of accessions regarding their
307 PA characteristics was independent of the environment. This, together with the high variability
308 observed for agronomic and quality traits, offers a unique opportunity for the improvement of sainfoin
309 through breeding.

310 PAs not only provide various benefits when fed to animals, they have also been shown to play an
311 important role in plant resistance to pathogens and pests (reviewed in Treutter 2006). In sainfoin,
312 concentrations of PA in the leaves were negatively correlated with the occurrence of sainfoin rust
313 (*Uromyces onobrychis* (Desmazières) Lévillé), indicating that PAs may play a role in sainfoin's
314 resistance to rust diseases (Malisch et al. 2016).

315

316 *Sainfoin – a PA plant with high potential*

317 The high variability observed in biomass yield, PA content and PA composition within and between
318 cultivars of sainfoin (Malisch et al. 2015) is characteristic for outbreeding forage grass and legume
319 species which are mainly bred through population improvement and the development of synthetic
320 cultivars consisting of many different genotypes (Posselt 2010). In order to optimally exploit the
321 bioactive properties of sainfoin, PAs in different quantities and of different structural characteristics
322 are needed. While it is commonly accepted that higher PD proportions and higher mDP (i.e. longer
323 polymer sizes) improve anthelmintic properties and influence rumen in vitro methane production
324 (Hatew et al. 2015; Novobilsky et al. 2013), their effect on other aspects of animal nutrition is poorly
325 understood. PAs with high PD proportions have been shown to effectively reduce rumen escape
326 proteins. Although desirable from an environmental point of view, PAs at too high concentrations
327 could lead to anti-nutritional effects by limiting protein use efficiency of the animal (Waghorn 2008).
328 Thus, in the future sainfoin with different PA composition are needed to fulfil specific demands for
329 bioactive effects. The large variability in PA content and composition present in sainfoin, which has
330 been shown to be rather stable across different environments, lays the basis for targeted improvement
331 and utilization in the future (Malisch et al. 2015; Malisch et al. 2016; Malisch et al. 2017).

332

333 *Genetic resources for a broad range of breeding targets*

334 The basic prerequisite for the wide adoption of a species in agriculture is the availability of well
335 adapted, high yielding cultivars. Consequently, targeted breeding for forage crop species has been
336 pursued for more than a century and resulted in numerous cultivars of major species such as perennial
337 ryegrass or alfalfa, adapted to a broad range of uses and environments (for overview see Boller et al.
338 2010a). In sainfoin, breeding activities have been rather scarce and the species has only quite recently
339 gained broader interest (Bhattarai et al. 2016). In Europe, only 22 sainfoin cultivars have been

340 reported by the European Commission in 2015, a very low number when compared to the 1,059
341 perennial ryegrass or 380 alfalfa cultivars reported (European Commission 2015). These cultivars
342 mostly originate from a few countries in Eastern and Southern Europe and may be often based on the
343 same breeding material as indicated by the similarity of their names (e.g., Vinovsk, Visnovsky and
344 Višňovský, or Perdix and Perly). A similar situation can be observed in North America, where
345 currently eight cultivars are registered and recommended for cultivation (Bhattarai et al. 2016). With
346 the increasing interest in sainfoin, plant breeding activities are likely to increase. For example, in
347 Canada a new cultivar has been recently released which is characterised by rapid regrowth and which
348 is persistent in mixtures with alfalfa (Acharya 2015).

349 So far, sainfoin breeding has been governed by goals common to most forage species, which include
350 high and stable biomass yield, persistence across a broad range of environments and high forage
351 quality. The variation observed for some of the key breeding targets is summarized in Table 1.
352 Germplasm native to the Near East is well adapted to dry and warm areas and has been shown to
353 clearly outperform cultivars from Western Europe when grown in the dry areas of the USA (Goplen
354 et al. 1991). Although successful cultivars with increased winter hardiness and spring vigour have
355 been developed for Canada (Goplen et al. 1991), there seem to be a general lack of cultivars well
356 adapted to cool season conditions. For example, in New Zealand, the lack of cultivars with sufficient
357 autumn growth and persistence seems to be the main reason for the limited importance of sainfoin
358 (Rumball and Claydon 2005). In Europe, the development of cultivars adapted to drought has been
359 successful, but there seems to be a lack of genetic variability for the adaptation to lower temperatures
360 and high soil humidity (Delgado et al. 2008; Liu et al. 2008). Thus, adaptation to wet and cold climates
361 remains one of the biggest challenges for sainfoin breeding. Other breeding goals include persistence
362 when grown with other species, as it is for example practised in Canada, where sainfoin is grown with
363 alfalfa mainly to reduce bloat (Sottie 2014), or resistance to pests and diseases (Piano and Pecetti
364 2010). In addition, quality traits such as content and composition of PAs or other phenolic compounds
365 have been emerging as additional breeding targets (Mueller-Harvey 2006). The high variation found
366 for these traits as reviewed above has indicated vast possibilities for improvement through plant
367 breeding. However, improving all these traits simultaneously remains challenging. For targeted
368 improvement of sainfoin in the future, diverse and well characterised genetic resources are needed.
369 Novel breeding approaches have to be developed based on knowledge on the mode of inheritance of
370 key breeding targets and the development of molecular genetic tools to assist phenotypic selection.
371 Due to its long history of cultivation, there are considerable amounts of genetic resources for sainfoin
372 available worldwide. Some of these resources are catalogued and made available through gene banks
373 such as the U.S. National Plant Germplasm System which lists 298 sainfoin accessions (GRIN 2016),
374 others are represented in local collections from diversity hotspots such as Iran (summarized in

375 Bhattarai et al. 2016). In Europe, gene pools can be clearly separated according to their geographic
376 origin (i.e. Western Europe, Eastern Europe and Asia) based on molecular genetic analyses
377 (Demdoum et al. 2012; Hayot Carbonero et al. 2012; Kempf et al. 2016), which is in accordance to
378 the geographic clusters as exhibited in their PA concentration and composition (Stringano et al. 2012).
379 In addition, high levels of diversity for agronomic and quality traits were observed within and among
380 sainfoin accessions from Spain (Delgado et al. 2008) and Iran (Mohajer et al. 2013; Table 1). The
381 high genetic variation available in sainfoin germplasm collections provides an invaluable resource
382 for further improvement of the species through targeted breeding.

383

384 **New knowledge for improved breeding strategies**

385 *Sainfoin – a highly self-fertilizing allogamous species*

386 In order to develop optimal breeding strategies, detailed knowledge on the mode of reproduction of
387 the species is indispensable. While autogamous (self-fertilizing) plant species are particularly suited
388 for the development of pure lines and hybrid cultivars, allogamous (cross-fertilizing) species are often
389 bred as population based cultivars, maintaining a high level of genetic diversity within cultivars,
390 which enables broader adaptation to a wide range of environmental conditions. Sainfoin is generally
391 considered to be an allogamous species, depending on insects for pollination (Bennett et al. 2001;
392 Tasei 1984). In contrast to other allogamous forage species such as perennial ryegrass or red clover,
393 where allogamy is secured through gametophytic self-incompatibility controlled by two or one
394 genetic loci, respectively (Do Canto et al. 2016; Riday and Krohn 2010), no such self-incompatibility
395 system has been described for sainfoin. Rather, allogamy is fostered by the flower architecture, with
396 the position of pistil and anthers largely preventing self-pollination (Knuth 1906; Özbek 2011). Such
397 a system is usually less effective in preventing self-fertilization when compared to gametophytic or
398 sporophytic self-incompatibility. Consequently, seed development after self-pollination has been
399 observed for sainfoin, confirming the possibility for self-fertilization in this species (Knipe and
400 Carleton 1972; Thomson 1938). A surprisingly high rate of self-fertilization of 48.5 to 64% was
401 observed in three F₁ populations obtained through targeted bi-parental bee-pollination in the
402 greenhouse, while rates of self-fertilization in multi-parental natural populations in the field were well
403 below 5% (Kempf et al. 2015). The high rates of self-fertilization observed in bi-parental populations
404 may be an effect of limited pollen availability in the glasshouse due to differences in flowering time
405 between the two parental plants. However, the results show that self-fertilization in sainfoin can be
406 quite frequent, which has direct implications on breeding practices. Polycross breeding schemes
407 (Posselt 2010) need to be composed of parental combinations with synchronous flowering time and
408 targeted pair-crosses may require the emasculation of maternal plants. Self-fertilization in allogamous
409 species is often accompanied by inbreeding depression, which results in a severe reduction of

410 agronomic performance. While this is particularly severe in species with strong self-incompatibility
411 such as perennial ryegrass (Kölliker et al. 2005) or red clover (Taylor and Anderson 1980), plant
412 height, vigour and seed yield were significantly reduced in sainfoin progeny derived from self-
413 fertilization when compared to progeny from cross-fertilization (Kempf et al. 2015). In a natural
414 tetraploid species such as sainfoin, inbreeding may be particularly pronounced through the loss of
415 tetra allelic interactions in the first few generations of inbreeding (Bingham et al. 1994; Gallais 2003).
416 Thus, although the possibility of self-fertilization in sainfoin may open new avenues for plant
417 breeding such as the development of inbred lines or hybrid breeding, care has to be taken to avoid
418 problems associated with severe inbreeding depression.

419

420 *New developments in molecular genetics and genomics*

421 Rapid developments in molecular genetics and genomics have generated a range of new tools and
422 strategies which have been shown to valuable complement phenotypic breeding approaches
423 (Varshney et al. 2005). Although novel approaches such as genotyping by sequencing (Elshire et al.
424 2011) may be readily applied to any species, most approaches require the availability of molecular
425 marker or genome sequence information for the species under study. As an orphan species, such
426 resources are scarce for sainfoin and most studies on genetic composition have been largely based on
427 universal marker systems such as internal transcribed spacer regions (ITS; Hayot Carbonero et al.
428 2012), inter SSR (reviewed in Bhattarai et al. 2016), SRAP markers (Kempf et al. 2015; Kempf et al.
429 2017) or sequence specific SSR markers derived from other species (Demdoum et al. 2012). In an
430 attempt to close this knowledge and resource gap, Mora-Ortiz et al. (2016) sequenced the
431 transcriptome of seven day old seedlings of five sainfoin genotypes from five different cultivars using
432 RNA-Seq (Wang et al. 2009). *De novo* assembly resulted in 92,772 transcripts, of which 63 were
433 annotated to be involved in the PA biosynthesis pathway (Mora-Ortiz et al. 2016). Interestingly, a
434 higher percentage of the PA biosynthesis related transcripts were expressed at high levels when
435 compared to all other transcripts, indicating a high expression of PA related genes even at very early
436 stages of plant development. These genes could be interesting targets when aiming to optimise PA
437 content and composition of sainfoin. Phylogenetic analysis revealed sainfoin to be closely related to
438 red clover which also has a haploid chromosome number of seven. However, it was also closely
439 related to *M. truncatula*, possibly due to genome duplication and tetraploidy (Mora-Ortiz et al. 2016).
440 Among the five sainfoin genotypes, 77,000 putative single nucleotide polymorphism (SNP) were
441 identified after stringent filtering. However SNP identification proved to be challenging, mainly due
442 to the tetraploidy of sainfoin and the lack of a reference genome which make the distinction between
443 homeologous and allelic SNPs difficult (Peralta et al. 2013). However, the RNA-seq approach
444 allowed for cost-effective identification of 3786 SSR loci (Mora-Ortiz et al. 2016). After testing 400

SSR primer combinations, Kempf et al. (2016) developed a set of 101 SSR markers which reliably detected polymorphism among a set of 32 sainfoin genotypes. The set contained genotypes from 29 different accessions, representing a broad range of geographical regions including the Near East, Eastern, Western and Southern Europe as well as the USA. With an average of 11.4 alleles per SSR locus detected across the entire dataset, these species specific sainfoin SSRs proved to be much more informative when compared to SSRs derived from other species (Demdoum et al. 2012). Cluster analysis allowed to separate the 32 sainfoin genotypes into two distinct groups, separating accessions for Western Europe and those from Eastern Europe and the Near East (Kempf et al. 2016). These SSRs provide a highly valuable source of markers for future genetic analyses in sainfoin.

First steps towards marker assisted selection

The improvement of the main breeding targets for sainfoin, such as biomass yield, increased persistence, PA content and composition and seed yield is rather difficult because phenotyping of these traits is usually labour and cost intensive and they are mostly controlled by multiple genetic loci. Thus, genetic gain through breeding is usually slow for such traits and for alfalfa yearly gains for dry matter yield lower than 0.2% y^{-1} have been observed over the past decades (Veronesi et al. 2010). In order to achieve genetic gains necessary to meet present and future demands on high quality forage legume cultivars, novel breeding strategies based on novel cultivar types, marker assisted selection strategies, the integration of field based and genome based selection as well as indirect selection strategies have been proposed (Annicchiarico et al. 2015). A common prerequisite for these strategies is a detailed understanding of the genetic control of the target traits, their interaction and their association with easily scorable molecular markers. Apart from a few studies on the variability of selected traits (for example Azuhwi et al. 2011; Delgado et al. 2008; Mohajer et al. 2011), information on response to selection or the genetic control of important traits in sainfoin is scarce. Kempf et al. (2017) investigated the variation of a range of agronomic traits and the PA content and composition in a segregating F_1 population derived from a bi-parental cross of two sainfoin plants. Even in such a narrow cross, the variability observed for traits such as vigour, plant height, seed yield, PA content, PC/PD ratio or individual flavanoids was large. Although some of the agronomic traits and some of the quality traits were correlated among each other, no clear correlation between agronomic and quality traits were identified which would allow to identify an easy target for indirect selection for PA content and composition (Kempf et al. 2017). However, one SRAP marker allele was identified to be significantly associated to the PD proportion, explaining 12.3% of the observed variation. In addition, several SRAP and SSR markers were observed to be associated with agronomic traits such as seed number $plant^{-1}$, plant height and vigour. These findings provide a first insight into

479 the genetic control of important traits in sainfoin and may serve, together with past and future
480 investigations, as the basis for the development of marker assisted breeding strategies.

481

482 **Conclusions**

483 Sainfoin is a valuable forage legume but underrepresented in today's agriculture mainly due to its
484 agronomic shortcomings and the limited availability of well adapted cultivars. Its ability to fix
485 atmospheric nitrogen together with its bioactive properties mainly due to its PA content, make it an
486 ideal component of sustainable grassland agriculture which has the potential to mitigate several issues
487 caused by agricultural intensification such as drug resistance of pests and pathogens, nitrogen losses
488 through rumen escape proteins and methane emissions by ruminant livestock. All these benefits have
489 increased the interest in sainfoin over the past years. Recent research has shown that substantial and
490 stable sainfoin yields can be achieved when grown in mixture with appropriate partner species. The
491 large variability in agronomic traits, PA content and composition identified within and between
492 different accessions, together with the fact that no significant genotype x environment interactions
493 were observed, lay the basis for targeted improvement of sainfoin cultivars with better agronomic
494 performance and with particular contents and compositions of PAs through breeding. This may be
495 further facilitated by recently gained knowledge on sainfoin's mode of reproduction together with the
496 newly developed genetic and genomic resources. Some of these findings will, in the future, have a
497 direct impact on the more widespread cultivation of sainfoin through the increased availability of
498 improved cultivars and recommendations about partner species and management practices. However,
499 in order to optimally utilize the bioactive properties of sainfoin, a more detailed understanding of the
500 mode of action of PAs and their individual components is needed. The rapid advances in the area of
501 analytical chemistry together with developments in molecular genetics and genomics will with no
502 doubt allow for rapid broad scale deployment of "sain foin" (healthy hay) in sustainable livestock
503 agriculture of the future.

504

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511

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Table 1: Variation for key breeding targets in sainfoin (*Onobrychis viciifolia* Scop.) germplasm (DMY = dry matter yield [t ha⁻¹], PH = plant height [mm], SY = seed yield [kg ha⁻¹], TF = time to flowering [days], CP = crude protein [g kg⁻¹ DM], NDF = neutral detergent fiber [g kg⁻¹ DM], ADF = acid detergent fiber [g kg⁻¹ DM], PA = proanthocyanidin [g kg⁻¹ DM], PDS = prodelphinidin share of CT [%])

Agronomic traits					Quality traits				Remarks	Reference
DMY	PH	SY	TF	CP	NDF	ADF	CT	PDS		
6.4-8.1	603-742	207-551	50-52						10 accessions	Mohajer et al. (2011)
11-13							17-24 ¹		3 accessions	Häring et al. (2008)
5.9-8.4				227-240	351-398	305-352			Rainfed, pure stands	Acharya (2015)
9.8-12.0				156-182	378-417	349-372	46.9-68.7 ¹		15 accessions	Azuhnwi et al. (2011)
							3.2-17.8 ²	48-74	6 accessions	Azuhnwi et al. (2013a)
0.5-8.0				224-234	211-246				CP, NDF: leaves	Borreani et al. (2003)
2.3-11.3				148-232	264-488				Pure stands	Liu et al. (2008)
3.9-7.6				157-198	374-489	283-370			Seasonal development	Turk et al. (2011)
9.3-378.3 ³	140-900		13-67						26 accessions	Zarrabian et al. (2013)
10.4-58.1 ³	376-459								10 accessions	Irani et al. (2015)
0.3-20.3 ³							23.0-47.5 ⁴	79-96	27 accessions; CT in leaves	Malisch et al. (2015)
	80-1290	0-4608 ⁵	19-65				9.7-47.2 ⁴	76-93	Segregating populations	Kempf et al. (2017)
				116-143	372-457	368-392				Parker and Moss (1981)
				121	478	433				Khalilvandi-Behroozyar et al. (2010)
				131-195	461-557	334-406	42.6-105.2 ¹		Seasonal development	Bal et al. (2006)
				114-177	431-476	343-433	41.9-99.5 ¹		5 accessions	Kaplan (2011)
				125-161		313-371	11.0-46.0 ¹			McMahon et al. (1999)

¹ HCl – buthanol method

² thiolysis method

³ single plants; g plant⁻¹

⁴ UPLC MS/MS method

⁵ seed number plant⁻¹