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NIRS for vicine and convicine content of faba bean seed allowed GWAS to prepare for marker-assisted adjustment of seed quality of German winter faba beans

NIRS für Vicin- und Convicin-Gehalt in Ackerbohnsamen ermöglichte eine GWAS als Vorbereitung einer marker-gestützten Adjustierung der Samenqualität von deutschen Winterackerbohnen

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Abstract

GWAS was applied to the antinutritive compounds vicine and convicine (V, C) in winter faba bean. V, C and V + C data for 189 inbred lines (five environments) were predicted by NIRS. These lines do not carry the strong “vc-“ allele (locus VC1). Lab data for 646 samples enabled our NIRS calibration, which performed well for V and V + C yet poor for C. Heritability was high (0.911; 0.868) for V and V + C and lower for C (0.737). From the 2542 mapped SNPs, 47 were significantly associated with V and one with V + C. Four SNPs mapped near to the VC1 locus and were significant for V. Seemingly, non-“vc-“ alleles at that locus contributed to V variation. Marker-assisted breeding with this germplasm can reduce the V + C content to about 0.44%, compared to the current lowest line with 0.55%. Further research will show inasmuch this can serve agronomy and breeding.

Keywords

Faba bean, *Vicia faba* L., Vicine, Convicine, NIRS, GWAS, QTL, breeding research

Zusammenfassung

Die antinutritiven Inhaltsstoffe Vicin und Convicin (V, C) in Samen von Winterackerbohnen wurden einer GWAS unter-

zogen. V-, C- und V + C-Werte von 189 Inzuchtlinien (fünf Umwelten) wurden mittels NIRS ermittelt. In diesen Linien kommt das starke „vc-“ Allel (VC1-Locus) nicht vor. Labor-Resultate von 646 Proben führten zu unserer NIRS-Kalibration, die gut für V und V + C tauglich war allerdings nicht tauglich für C. Die Erbllichkeit war hoch für V und V + C (0,911; 0,868) und niedriger für C (0,737). Von den 2542 kartierten SNPs waren 47 signifikant mit V und einer mit V + C assoziiert. Vier SNPs, die nahe beim VC1-Lokus kartierten, waren für V signifikant. Anscheinend trugen nicht-„vc-“ Allele an diesem Locus zur V-Variation bei. Markergestützte Züchtung in diesem Genpool kann die V + C-Gehalt auf etwa 0,44 % reduzieren, im Vergleich zur aktuell niedrigsten Linie mit 0,55 %. Weitere Forschung wird zeigen, wie diese Ergebnisse der Agronomie und Züchtung dienlich sein werden.

Stichwörter

Ackerbohne, *Vicia faba* L., Vicin, Convicin, NIRS, GWAS, QTL, Züchtungsforschung

Introduction

Vicia faba L., faba bean, is a grain legume grown as row crop in most temperate and semi-arid regions of the Old World; and, after Christopher Columbus and James Cook, in the An-



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dean countries and across Australasia. It is barely found in tropical regions such as South-East Asia (Hawtin & Hebblethwaite, 1983; Cubero, 2011). Besides condensed tannins and several less important antinutritive compounds (saponins, trypsin-inhibitors, α -amylase inhibitors, lectins), the antinutritive compounds vicine (V) and convicine (C) are of high relevance to the use of faba bean seed as feed (Frauen et al., 1984a; b; Khazaei et al., 2019; Nolte et al., 2020a; b) for pork, poultry and further non-ruminants. Moreover, V and C are a point of concern for people with a genetic deficiency of their X chromosome-located G6PD-enzyme. This deficiency is a serious issue in regions with historic or current threat of Malaria. Ingestion of V and C may cause serious health disturbances, called favism; yet this has an intricate protective connection to Malaria. Vicine and convicine are glycosides, they contain pyrimidine bases and glucose. They co-occur in faba bean. Upon ingestion, they are hydrolysed into their aglucones, which are the antinutritive and favism-causing molecules (Farhud & Yazdanpanah, 2008; Lessire et al., 2017; Luzzatto & Arese, 2018). Vicine and convicine occur nearly exclusively in *Vicia* species (Khazaei et al., 2019). Their sum content (V + C) in faba bean seeds shows a continuous variation, caused by genetic and environmental effects (Duc et al., 1989; Puspitasari, 2017). V + C varies from about 0.3% to 1.4% in seed dry matter (Björnsdotter et al., 2021; Bjerg et al., 1985; Lattanzio et al., 1983; Pulkkinen et al., 2015; Puspitasari, 2017; Duc et al., 1989, Khamassi et al., 2013; Frauen et al., 1984a; b). V and C also occur in roots, leaves, and shoots (Sharifi, 2021; Tacke, 2021).

Currently, breeders use the “vc-“allele to drastically reduce V + C. This allele was found by Duc et al. (1989) in the gene bank accession 1268(4)(1). When homozygous, this allele reduces the seed content of V + C to values between about 0.02 and 0.06%, whilst heterozygotes have intermediate levels (Duc et al., 1989; Khazaei et al., 2019; Tacke et al., 2021). The V + C content of mature seeds reflects the genetic status of the mother plant, not of the seeds. Plants homozygous for “vc-“ show their very low content not only in their grains but as well in roots and shoots (Tacke, 2021).

Recently, the “vc-“allele was identified as a variant of the bi-functional enzyme RibA (VC1), which catalyzes a purine metabolic pathway towards V and C; the sequence and mapping location of the gene and DNA-markers for marker-assisted selection are now known (Björnsdotter et al., 2021; Tacke et al. 2021).

Bjerg et al. (1984) reported inhibitory effects of convicine, vicine and L-dopa (all occurring in faba bean seed) on growth rate of *Botrytis cinerea* and *Aschochyta faba*, yet did not fully disentangle their effects (cf. Ray & Georges, 2010). Based on bioassays with the fungi *Fusarium colmorum*, *Alternaria alternata*, *Cladosporium herbarum* and *Botrytis cinerea*, Pavlík et al. (2002) reported an important fungicide effect of vicine. Hence, “vc-“ homozygous faba beans may be more susceptible to fungal pathogens than wild-type beans. Interestingly, Griffiths & Ramsay (1992) and Griffiths & Ramsay (1996) reported relatively high contents of V + C in the roots of germinating faba beans, as supported by the findings of Sharifi (2021). Tacke (2021) did not, however, in a series of field tri-

als, substantiate an increased susceptibility of low V + C genotypes to soil-born fungi.

Reports on the impact of V and C on non-ruminants such as chicken are ambiguous. Nevertheless, diets with contents of V + C less than ca. 0.1% seem to be well tolerated by poultry (Nolte et al. 2020a; b; Lessire et al. 2017; Laudadio & Tufarelli 2010; Fru et al. 2007). Those studies that resulted in clear problems (reduced egg and yolk weights) dealt with 0.4% or more V + C in the chickens’ diets (Olaboro et al. 1981; Muduuli et al. 1981). Non-“vc-“ faba bean, with its 0.3% to 1.4% content of V + C, is usually mixed into diets with a proportion of only 10%-20% (Farrell et al., 1999; Bellof et al., 2016). Therefore, faba beans with only moderately reduced V + C content may already well comply with the requirements of poultry.

There may be a potential conflict of aims: pro very low V + C in favour of high feed value; or against very low V + C in favour of resistance against fungi. The question arises whether, avoiding the “vc-“ allele, the wild-type variation for V + C could be exploited to breed for a level of V + C which is low enough so as not to significantly affect feed quality yet high enough to not put resistance to pathogens at risk without need. Consequently, here we analyze the quantitative-genetic variation of the content of V and C in wild-type material.

Materials and methods

Genotypes

This study was based on 189 lines, inbred to F_{10} or beyond, which were derived via single seed descent from the Göttingen Winter Bean Population (Link & Arbaoui, 2006). This is a well-known, winter-hardy faba bean breeding germplasm. These 189 lines (the A-set lines), have been analyzed for auto-fertility, *Ascochyta* resistance and drought and frost tolerance (Puspitasari, 2017; Ali et al., 2016; Faridi et al., 2021). The above-mentioned „vc-“ allele is not present in this material, as proven by its range of V + C values (see below) and from its breeding history (Link & Arbaoui, 2006).

DNA-Markers

In cooperation with O’Sullivan and Angra at Reading (UK), the A-set lines were genotyped for 2.542 genetically mapped SNPs (O’Sullivan et al., 2019; cf. Ali et al., 2016). This was based on their 50K SNP Affymetrix chip “Vfaba_v2”. The genetic map was constructed from 82 genotyped F_6 -RILs from the cross NV644 (Kasztelan, a low-protein type) \times NV153 (IG 12658, a dwarf type; Webb et al., 2016).

Phenotypic data, analyses of variance

Mature seed from the 189 A-set lines were used to study their content of V and C. Seed was harvested from plants grown in pollinator-proof houses in the seasons 2013, 2014 and 2019 (Puspitasari, 2017). In addition, seed samples of combine-harvested field plots from seasons 2013 and 2017 of these lines were used. All five experiments were conduct-

ed at Göttingen, Germany, as randomized complete block trials with two replicates. From the theoretical total of 1,890 seed samples, 1,662 were available. For these, their contents (% in dry seed matter) of V, of C, and of V + C were predicted based on NIRS (near infrared reflectance spectroscopy; Duc et al., 1989; Font et al., 2006). The NIRS-predicted values were statistically analyzed, with the factors “inbred line”, “environment” and “replicate” as random factors. Significance of genetic variance between the lines was examined by means of F-test. Broad sense heritability (h^2) was expressed as ratio between a trait’s variance component due to the inbred lines and the phenotypic variance among the lines (using PLABSTAT; Utz, 2011).

NIRS calibration

To develop a predictive NIRS-based model, a total of 659 seed samples were analyzed using seed lots from 2013 until 2019, from the pollinator-proof houses and the field plots. The majority of these seed lots were from the A-set lines and from few additional lines with similar pedigree. Yet, one hundred samples from lines homozygous for “vc-”, and thus very low in V and C, were used for making this calibration. The purpose was to tune the calibration and to focus its validity especially to the low range of V + C. Dry samples were ground with a Retsch ZM100 mill to a particle size of 0.5 mm (Retsch GmbH, Haan, Germany). In Saskatoon, Canada, 300 of the samples were analyzed for V and C by flow injection analysis – mass spectrometry using an ion mobility interface (i.e., FIA-FAIMS-MS; Purves et al., 2017). Results from FIA-FAIMS-MS are comparable with a hydrophilic interaction liquid chromatography developed by the same group (Purves et al., 2018), but the former method was used here because it is more rapid (about 1 vs. 10 mins/analysis) and thus suitable for larger numbers of samples. The remaining 359 samples were analyzed at NIAB, UK. There, the analyses followed Khamassi et al. (2013) with slight adaptations (HPLC). Duplicate samples of 0.5 g bean flour were water-extracted and sonicated for 10 minutes and allowed to settle overnight at 4°C. A 1 ml aliquot was centrifuged at 14000 rpm for 15 minutes and filtered (0.2micron) for analysis. A Dionex Ultimate 3000 HPLC was used with a Kinetex 5 μ m C18 100A column (Phenomenex). The mobile phase was 100% phosphate buffered water (pH 7) with flow rate of 1.5 ml/min. The total run time was 10 mins. V and C peaks were detected at 280 nm.

To combine the FIA-FAIMS-MS and HPLC data, 32 samples were analyzed at Saskatoon as well as at NIAB. The data as reported from NIAB was transformed to match the FIA-FAIMS-MS scale. The correlations for the results between the two labs were $r=0.953$ for V, $r=0.976$ for C, and $r=0.947$ for V + C ($P=1\%$). The data at the FIA-FAIMS-MS scale for these 32 samples varied from $0.05\% < V + C < 1.03\%$. The NIAB data was accordingly multiplied by 1.3394 for V, by 0.9480 for convicine and by 1.2374 for V + C to transform it to the FIA-FAIMS-MS scale.

The values (at FIA-FAIMS-MS scale) of all 659 calibration samples ranged for V from 0.005% to 1.028% and for C from 0.000% to 0.362%. The sum, V + C, ranged from 0.006% to

1.229%. In this calibration set of samples, the correlation between V and C was $r = 0.394$ ($P=1\%$).

NIR spectra of these calibration samples, and all others, were recorded with NIR system model 6500 monochromator instrument (NIR SYSTEMS, INC., Silver Spring, Maryland, USA). Reflectance spectra data ($\log 1/R$) was collected between 408 and 2498 nm. Lab data and NIR spectra were employed to develop a calibration using the Modified PLS method in the WinISI II software (WinISI II, Infrasoft International, Hosham, West Sussex, UK). NIRS spectra are by default treated mathematically to smooth data. WinISI II specifies the derivative order (first or second derivative of the $\log 1/R$ data), the number of data points (segment length) over which a derivative is taken (so-called gap), the smoothing segment length for a first and second smoothing (Shenk et al., 2007). After following a try-and-search strategy to optimize setting of the parameters (Puspitasari, 2017), the first derivative was used, and for gap, the first and the second smooth the values were 4, 4, 1, respectively. The final calibration model was developed using 12-fold cross-validations, a maximum of 16 terms was allowed. This final calibration model was based on 646 samples, 13 samples were eliminated as outliers, following the default procedure of WinISI II.

Predicting V, C, V + C data based on NIRS

The 1662 samples mentioned above from the 189 A-set winter faba bean lines produced in the five environments were ground and used to record their NIR spectra. From these spectra, values for V, for C, for V + C were predicted to enter analyses of variance. The sum of V + C was predicted from the specific calibration for this sum.

GWAS

Following Honsdorf et al. (2010) and Ali et al. (2016), simple means for V, C and V + C contents of each A-set line across environments as computed by PLABSTAT were used as their phenotypes for GWAS. After filtering out SNPs with a minor allele frequency of $<5\%$, 2,400 SNP markers remained for GWAS (TASSEL3.0.170; mixed linear model; optimum level of compression, re-estimation of variance component estimates for each marker; Bradbury et al., 2007). Heterozygous markers were coded as missing data (average degree of marker heterozygosity was 0.98%). The kinship matrix calculated by TASSEL, using the filtered SNP, was employed. The significance of the SNPs was established with a FDR of 20% (Benjamini & Yekutieli, 2005). The difference between the means of the two marker classes was defined as the phenotypic marker effect (Bradbury et al., 2007). Analysis of gamete phase disequilibrium (LD) was performed. LD was expressed as R^2 (Flint-Garica et al., 2003).

A marker score for each A-set line was calculated for V by adding up the GWAS-predicted effects of the significant SNP, with due consideration of positive or negative sign. An alternative marker score was calculated by fixing the effect sizes to 1.0, hence adding up “+1” or “-1”, depending on positive or negative effect sign.

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Results

The NIRS calibration algorithm was based on 646 samples. The coefficients of determination between lab-values and NIRS-predicted values were $R^2 = 0.948$ for V, $R^2 = 0.508$ for C, and $R^2 = 0.937$ for V + C. When focusing on the samples with NIRS-predicted V values higher than 0.25% (Fig. 1), to approximately fit to A-set line data, then the value was

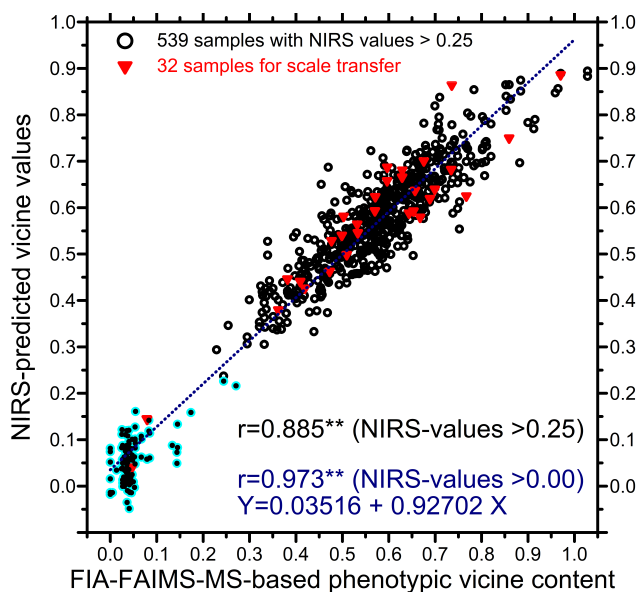


Fig. 1. Correlation between FIA-FAIMS-MS based vicine values and NIRS-predicted values for the entire set of 646 samples and for the 539 values predicted to be higher than 0.25%.

$R^2 = 0.783$ (all significant at $P=1\%$). To describe the validity of this calibration algorithm, standard errors of cross validation (SECV) and coefficients of determination of cross validation (R^2CV) were calculated: SECV = 0.060% and $R^2CV=0.931$ for V; SECV=0.056% and $R^2CV= 0.385$ for C; SECV = 0.078% and $R^2CV= 0.920$ for V + C).

The ranges from minimum to maximum value in the 1,662 raw data from the 189 lines (from five environments, two replicates each) were 0.257% to 0.988% for V, 0.002% to 0.323% for C, and 0.362% to 1.222% for V + C. No lower results occurred, as expected, since the “vc-“allele is absent in this material. When calculating the 189 inbred line means across the five environments, V varied from 0.413% up to 0.846% (general mean was 0.593%); C varied from 0.066% to 0.217% (general mean 0.132%). The sum, V + C, varied from 0.547% to 0.953% (general mean was 0.726%). Vicine and convicine were uncorrelated ($r= 0.070$). The correlation between V and V + C was $r=0.952$ ($P=1\%$).

Analyses of variance of these results (Table 1) revealed a broad a sense heritability of $h^2 = 0.911$ for V, and $h^2 = 0.887$ for V + C, and of only $h^2 = 0.737$ for C. The corresponding F-tests showed high and highly significant ($P=1\%$) results of the genotypes ($3.8 < F < 11.3$). It was thus deemed appropriate to undertake further genetic analyses.

The correlation between the NIRS-predicted value for V + C and the sums of the NIRS-predicted V plus the NIRS-predicted C was $r=0.98$ ($P=1\%$). The former is reported in Table 1 and used for GWAS.

Table 1. Analysis of variance for NIRS-predicted data on vicine, convicine and V + C

Environments	Degrees of freedom	Mean Squares	Variance components	F-value*	Standard error
Vicine content (%)					
Genotypes	188	0.0647	0.0059	11.29	0.0239
Environments	4	2.2370	0.0059	390.1	0.0039
G × E	655	0.0057	0.0021	3.79	0.0275
Error	815	0.0015	0.0015		
$h^2 = 0.911$; mean across genotypes: 0.5933%					
Convicine content (%)					
Genotypes	188	0.00711	0.00053	3.80	0.0137
Environments	4	0.08347	0.00022	44.53	0.0022
G × E	655	0.00187	0.00038	8.44	0.0105
Error	815	0.00022	0.00023		
$h^2 = 0.737$; mean across genotypes: 0.1324%					
V + C content (%)					
Genotypes	188	0.0704	0.0061	7.58	0.0305
Environments	4	1.9706	0.0052	212.01	0.0050
G × E	655	0.0093	0.0034	3.59	0.0360
Error	815	0.0026	0.0026		
$h^2 = 0.868$; mean across genotypes: 0.7123%					

*all F-values significant at $P = 1\%$

A total of 2.400 SNP markers were available for GWAS. The average LD between these markers was $R^2 = 0.0081$, similar to the value reported by Ali et al. (2016; $R^2 = 0.0077$) for the same set of lines. The average LD between the significant markers (see below) was $R^2 = 0.0116$, the maximum value was $R^2 = 0.787$. As example for LD decay along cM distances, the pattern on chromosome 1 is displayed. The distances between the markers were taken from their positions on the genetic map of the NV644 × NV153 cross, 586 SNPs were accordingly mapped on chromosome 1. The average distance of the 209 marker pairs that showed LD values of $R^2 > 0.25$ was 2.58 cM. Ten of these marker pairs were more distant than 10cM (Fig. 2).

Table 2 shows the significant SNP (FDR=20%). Of the 47 significant SNP for vicine, one was significantly associated with V + C. When, alternatively, applying FDR=10%, then two of the 47 SNP (Affx-309732154 and Affx-310628027) were declared significant for V, and none for V + C. LD-based clusters of markers are revealed based on a minimum LD of $R^2 = 0.07$ (Table 2).

The SNP associated with the largest effect on V explained 11.7% of the phenotypic variance and a 0.056% difference between marker classes, with 160 inbred lines homozygous for C:C showing the higher mean V value. The effect sizes and R^2V were barely correlated ($r=0.08$).

The sum of all 47 SNP-effects amounts to 1.49% of V which is higher than the content of the line W-159 which had the maximum V value (0.846%). The sum of the 47 values for their R^2V was 336%. Keeping only that SNP per cluster with the largest effect (excluding markers 1 & 2 & 3; 7 & 8 & 9; 27 & 28 & 32; 46 & 47) reduced these values to some extent: to 1.15% as sum of effects and to 264% as sum of R^2V .

When correlating the marker scores of the 189 lines with the phenotypic result of V, the coefficient was $r = 0.775$ ($P=1\%$).

This correlation was slightly smaller ($r=0.721$; $P=1\%$) when calculating the alternative marker score (the same effect size for all markers).

Discussion

In the EU and abroad, consumers appreciate local production, sustainable agronomy and livestock health (FAO, 2017). This promotes the use of feed stuff such as faba bean, as substitute for imported soy bean meal and meal of mineral-N fertilized rapeseed or sunflower. The notion that maximum performance of livestock must be achieved, even if this comes with ethical or ecological concerns, is challenged. Domestic pulses as local and traditional feed stuff see political support (BLE-BÖLN, 2020; ARC, 2020).

Here, we work on faba bean, a local and traditional pulse crop. We developed tools to genetically reduce the content of vicine and convicine in German winter faba bean grains without bringing it to near zero, based on the conjecture that a certain level of these antinutritive ingredients may be biologically useful. However, this notion deserves further research.

The seed content of V + C in genetically low V + C cultivars (homozygous for “vc-”) is about 0.02 to 0.06% (Björnsdotter et al., 2021; Tacke et al., 2021; Duc et al., 1989); whereas the lowest A-set line showed 0.547%. A 20% dose of such faba bean in feed stuff (Farrell et al., 1999) would lead to 0.11% V + C in the diet. This content did not have adverse effects on chicken performance in the experiments of Nolte et al. (2020a;b). The genetics of the A-set lines promises successful breeding towards even lower values without employing the “vc-” allele, supposedly reaching a compromise level meeting both objectives: high feed quality and not putting resistance at risk.

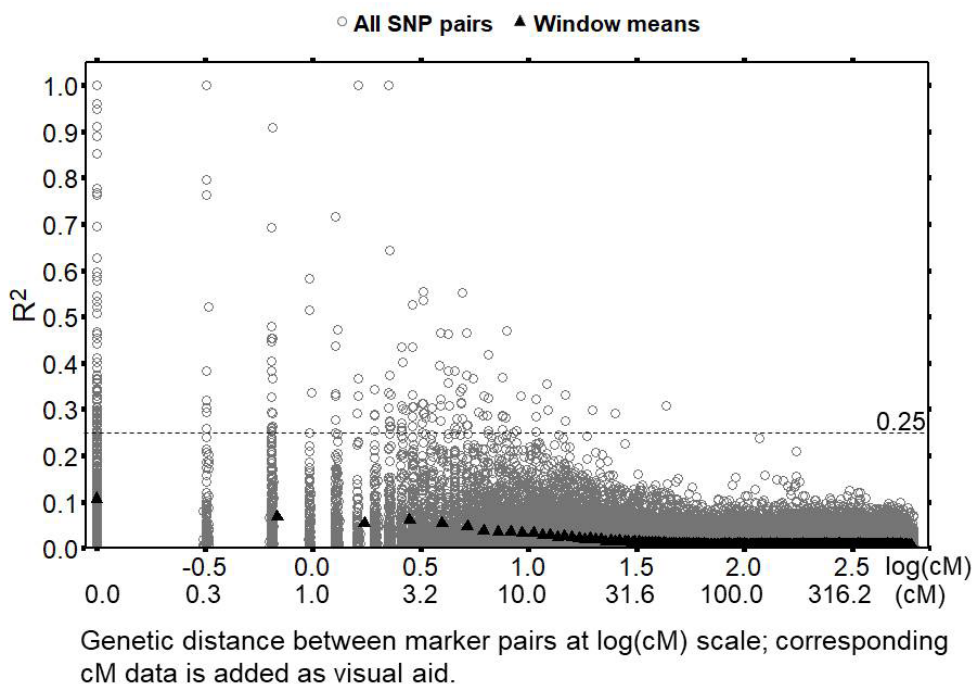


Fig. 2. Decay of LD of the 171.405 marker pairs on chromosome 1 (NV644 × NV153 linkage map). The average R^2 values of windows with 605 marker pairs (collecting all pairs at 0cM in one window) are indicated.

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| **Table 2.** GWAS for V and VC (% in seed dry matter) in 189 German winter faba bean lines

SNP Number (LD-Cluster)*	SNP "Affx"-	Chromosome [§]	Position (cM) [§]	P-value	Effect**	Polymorphism; increase-allele; No. lines***			R ² V [†] (%)
Content (% of seed dry matter) of vicine plus convicine (as predicted via NIRS)									
33	Affx-310027095	4	106.18	1.43 10 ⁻⁰⁴	0.0480	A/G	G	94	9.12
Content (% of seed dry matter) of vicine (as predicted via NIRS)									
1(1)	Affx-309161521		0.98	1.41 10 ⁻³	0.0395	T/C	T	150	7.01
2(1)	Affx-309473691		0.98	1.38 10 ⁻³	0.0392	T/G	G	143	7.04
3(1)	Affx-1003937842		3.87	1.41 10 ⁻³	0.0419	A/G	G	51	7.17
4(1)	Affx-1003954634		3.87	2.19 10 ⁻⁴	0.0431	T/C	C	97	5.93
5	Affx-310640326		106.11	3.60 10 ⁻⁴	0.0292	T/C	T	125	8.39
6(2)	<u>Affx-309732154</u>		220.19	1.32 10 ⁻⁵	0.0561	T/C	C	160	11.71
7(2)	Affx-310761670		225.08	2.66 10 ⁻³	0.0200	T/C	T	97	6.35
8(2)	Affx-308714105	1	230.28	7.09 10 ⁻⁴	0.0188	G/A	A	135	7.88
9(2)	Affx-310049683		242.58	5.78 10 ⁻⁴	0.0388	T/G	G	95	7.93
10	Affx-310717580		309.93	1.72 10 ⁻³	0.0311	A/G	G	159	6.79
11	Affx-310401178		403.71	8.36 10 ⁻⁴	0.0084	A/G	A	145	7.59
12	Affx-309036234		435.91	3.36 10 ⁻³	0.0171	T/C	T	124	6.13
13	Affx-309615802		493.01	1.78 10 ⁻³	0.0110	T/C	C	141	6.83
14	Affx-308989324		508.89	9.00 10 ⁻⁴	0.0202	A/G	G	59	7.53
15*	Affx-309170690		532.43	1.83 10 ⁻³	0.0436	A/G	G	85	6.79
16	Vf_Mt4g016930		21.40	2.87 10 ⁻³	0.0474	A/G	G	30	4.72
17	Affx-309903736		61.80	2.77 10 ⁻³	0.0120	A/G	G	71	6.45
18	Affx-309970506	2	123.92	7.86 10 ⁻⁴	0.0299	A/G	A	69	7.64
19	Affx-309859410		148.89	1.62 10 ⁻³	0.0448	T/C	C	136	6.88
20	Affx-308983861		160.62	8.94 10 ⁻⁴	0.0125	T/C	C	48	7.61
21	Affx-310060263		3.21	2.28 10 ⁻³	0.0210	A/G	G	35	6.50
22	Affx-310730983		8.08	2.71 10 ⁻³	0.0431	A/G	A	77	6.45
23	Affx-310504492	3	112.47	1.30 10 ⁻³	0.0409	T/C	C	136	7.14
24	Affx-308750155		133.11	2.37 10 ⁻³	0.0236	T/C	C	98	6.47
25	Affx-309708615		153.56	7.75 10 ⁻⁴	0.0037	T/C	C	104	7.79
26	Affx-310540370		6.76	2.03 10 ⁻³	0.0239	A/G	G	114	6.63
27(3)	Affx-309815489		33.56	1.51 10 ⁻⁴	0.0366	T/C	T	97	9.27
28(3)	Affx-309712729		35.83	1.07 10 ⁻³	0.0292	T/C	T	112	7.29
29	Affx-310324523		46.56	5.75 10 ⁻⁴	0.0401	C/G	C	69	7.94
30(3)	Affx-309866454		53.36	8.26 10 ⁻⁴	0.0484	T/G	G	119	7.57
31	Affx-308743991	4	86.40	1.14 10 ⁻³	0.0535	A/G	A	24	7.23
32(3)	Affx-310120776		86.40	6.27 10 ⁻⁴	0.0353	C/G	G	120	7.84
33	Affx-310027095		106.18	3.92 10 ⁻⁴	0.0311	A/G	G	94	8.35
34	Affx-308861851		113.33	3.50 10 ⁻³	0.0207	A/G	G	104	6.16
35	Affx-309691880		128.55	2.51 10 ⁻³	0.0184	T/C	T	108	6.44
36	Affx-308653794		145.86	1.60 10 ⁻³	0.0375	A/G	G	107	6.89
37	Affx-309747295		161.87	1.30 10 ⁻³	0.0348	T/C	T	21	7.09
38	<u>Affx-310628027</u>		17.46	8.15 10 ⁻⁵	0.0361	A/G	G	16	9.90
39	Vf_Mt7g101170	5	92.84	1.73 10 ⁻³	0.0507	C/T	T	156	5.21

For this breeding objective, NIRS is possibly a very adequate approach. In the calibration samples, the ranges (maximum minus minimum) were 1.023% for V, 0.362% for C and 1.223% for V + C. Correspondingly, the ratios of these ranges to their

SECV (Fearn, 2002) were RER=17.1 for V, RER=6.5 for C, and 15.7 for V + C. Williams & Sobering (1993) ascertain that RER=10 is the lower limit for meaningful NIRS-based screening. Thus, the RER value for C here is unsatisfactory, as already

Table 2. Continued

SNP Number (LD-Cluster)*	SNP "Affx"-	Chromosome [§]	Position (cM) [§]	P-value	Effect**	Polymorphism; increase-allele; No. lines***		R ² V [†] (%)	
40	Affx-310352731		25.89	1.76 10 ⁻³	0.0367	T/C	C	169	6.77
41	Affx-308481800		33.06	2.69 10 ⁻³	0.0220	G/A	A	110	6.37
42	Affx-310302620		106.18	2.96 10 ⁻³	0.0134	T/C	C	33	6.30
43	Affx-308848038	6	146.58	1.51 10 ⁻³	0.0332	T/G	G	87	6.94
44(4)	Affx-308323030		161.80	1.97 10 ⁻³	0.0560	T/G	T	16	6.71
45	Affx-309693598		165.92	3.69 10 ⁻⁴	0.0134	A/G	A	46	8.56
46(4)	Affx-308386489		171.56	1.06 10 ⁻³	0.0385	T/C	C	66	7.42
47(4)	Vf_Mt4g053880		178.90	2.08 10 ⁻³	0.0418	G/A	A	46	5.05

The two underlined SNP are significant even with FDR 10% (see text);

*LD expressed as R² (values in bracket) revealed four LD clusters:

Cluster 1 between marker pairs 1 & 2 (0.787), 2 & 4 (0.071), 3 & 4 (0.412);

Cluster 2 between marker pairs 6 & 7 (0.153), 6 & 8 (0.172), 7 & 8 (0.080), 7 & 9 (0.073);

Cluster 3 between marker pairs 27 & 28 (0.236), 30 & 32 (0.070);

Cluster 4 between marker pairs 44 & 46 (0.090), 44 & 47 (0.193), 46 & 47 (0.092).

[§]Map position according to Angra and O'Sullivan (2021; in preparation);

**Difference between the means of the two marker-classes of inbred lines;

***Indicates marker-allele associated with the higher phenotypic value and number of lines having such allele;

[†]Percent of phenotypic variance explained.

seen from R²CV=0.385. Obviously, this low performance of the calibration for C contributed to its relatively low heritability (Table 1). It probably also accounts for low correlation between C and V compared to that found in the calibration set of samples ($r=0.070$ vs. $r=0.394$). Furthermore, the poor calibration for C might be the reason for finding only one significant SNP for V + C, although 47 significant SNP were identified for V. The SNP that was in common for V + C and V (marker number 33, Table 2) showed a smaller P-value ($1.43 \cdot 10^{-4} < 3.92 \cdot 10^{-4}$) for V + C than for V.

A large investment to predict V and C via NIRS has been made here. The majority of the samples employed to calibrate (546 out of 646) represented the genotypes and environments of the 1.662 samples, for which V, C, and V + C had to be predicted. The NIRS-application should be highly valid, because of that marked overlap of the samples used to develop the calibration and the NIRS-predicted samples. The high heritabilities ($h^2 = 0.911$ for V; $h^2 = 0.868$ for V + C) support this notion. The failure to reliably predict C may be caused by the very low percentage of that ingredient (from 0.000% to 0.362% in the calibration samples).

No investigation was carried out to compare the current NIRS algorithm with what would have resulted if the 100 "vc-" samples had not been included in the calibrations set of samples. With all except one significant marker found only for V, we can only speculate whether marker-based decreasing V instead of V + C may make a difference.

For V and for V + C, NIRS was efficient, in contrast to the very early attempt to predict V and C based on NIRS by Duc et al. (1989). These authors concluded that NIRS was only useful to identify very extreme V and V + C values. Yet, application of our calibration to different faba bean germplasm and seed lots from different germplasm and production environments would be expected to worsen its performance.

When adding up the effects of all 47 SNPs, ignoring mutual LD between several of them, we obtained unrealistic results. With the applied FDR of 20%, about nine of the SNPs are expected to be false positives, and with the limited number of lines (N=189), QLT effects are at risk to be overestimated (Josephs et al., 2017; Vales et al., 2005; c.f. Göring et al., 2001). Hence, adding up the 47 effects is naïve. The reduction of the supposedly explained phenotypic variance from R²V=336% to R²V=264% by excluding several SNPs based on mutual LD indicates that the number of detected QTL is probably lower than 47.

When applying the linear regression (Fig. 3) between the phenotypic values and the marker score calculated with same

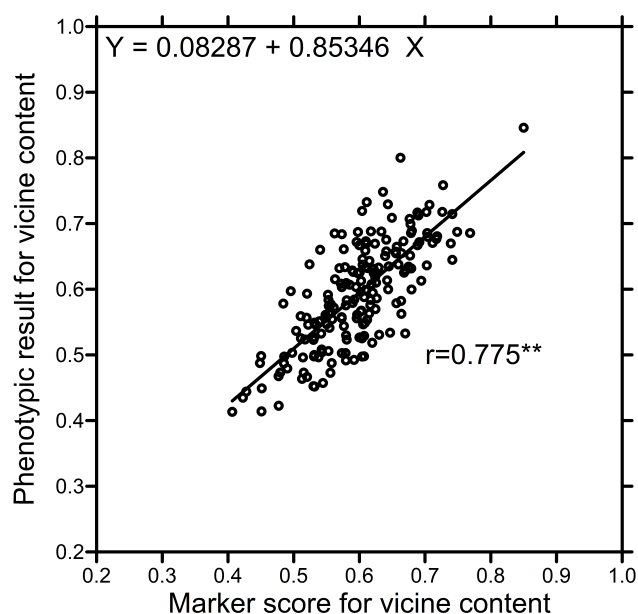


Fig. 3. Correlation between marker scores of the 189 A-set lines and their phenotypic values for vicine content

effect size for all SNPs, realistic and presumably useful predictions occur. The small increase of the correlation coefficient from Fig. 4 to Fig. 3 ($0.775 > 0.721$) implies that very little information lies in the variation of the 47 estimated effect sizes. Genetically pyramidizing all decrease or all increase alleles in the same inbred line promises inbred lines with 0.23% and 0.94% of vicine content. It would need several cycles of recombination and segregation to meet this objective.

The four SNPs at 0.98 and 3.87 cM of chromosome 1 (Table 2; cluster 1) may be associated with the VC1 locus. Ecke (unpublished; *c.f.* O'Sullivan & Angra, 2016) has shown that they are in a region with well-defined synteny between *Vicia faba* and *Medicago truncatula* (*Mt*). Markers 1 & 2 and 3 & 4 are located at about 337.6kb and 1,319.1kb, respectively, of chromosome 2 of the *Mt* reference sequence (LIS, 2021). Their distance of about 2.9cM corresponds to 982kb in *Mt*., making a ratio of 339kb/cM. Björnsdotter et al. (2021) have localized the VC1 gene at 1,850.3kb on chromosome 2 of *Mt*., about 531kb after markers 3 & 4. This would place the VC1 gene on the genetic map of *V. faba* about 1.57 cM after these two markers, close enough (Fig. 2) to allow for marked LD between cluster 1 and the VC1 locus. Although "vc-" is not present here, more than one wild-type allele may be present in the A-set lines at the VC1 locus and cause a traceable contribution to the genetic variation of V.

The strongest marker, marker 6 (Table 2; Affx-309732154), finds its syntenic position on *Mt* chromosome 5 at about 1,690.2kb, as part of a plant/T7N9-9-protein-annotated gene. This and the inspected twelve genes on either side, from 16,742kb to 16,964kb in *Mt*, are not directly discernable as candidate genes, although at 16,763kb and at 16,936kb, two transcription factors were found. In short time, a physical sequence of the faba bean genome will become available, allowing more straightforward analyses (Adhikari et al., 2021).

To directly prepare a marker-assisted breeding, marker-based low V + C lines and marker-based complementary

A-set lines were sought. The A-set line S_034 ($V=0.4133\%$; $V + C=0.5628\%$) was found to possess the decrease allele for V at 33 of the 47 SNPs, and the line S_295 ($V=0.4349\%$; $V + C=0.5574\%$) at 31 of the 47. Since the overlap is small, the F1 (S_034 × S_295) would be either homo- or heterozygous "decrease" at 42 of the 47 SNP loci. Since this was the best case, the lowest predicted V content after one breeding step with this cross would be a line with 42 of 47 SNP with the decrease allele for V, hence a predicted value of 0.31% V and correspondingly a predicted content of V + C of about 0.44%. This might be compared to the current lowest phenotype for V with 0.4133% (line S_034) and for V + C at 0.5473% (line S_172). Further research will show whether these results suffice for agronomic and feed quality purposes.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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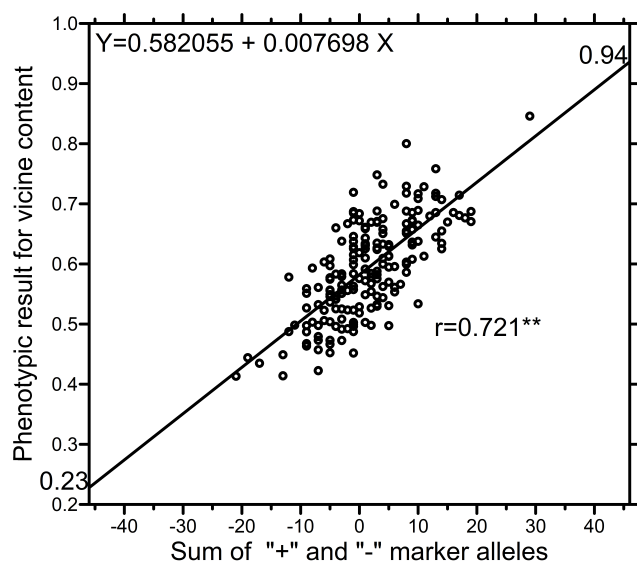


Fig. 4. Correlation between marker scores of the 189 A-set lines calculated with same effect size for all SNPs and their phenotypic values for vicine content

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