Genotypic variation in leaf epicuticular wax quantity in a large faba bean (*Vicia faba* L.) germplasm collection

Hamid Khazaei[1,2*], Arja Santanen[1], Kenneth Street[3], and Frederick L. Stoddard[1]

[1] Department of Agricultural Sciences, Viikki Plant Science Centre, Helsinki Sustainability Science Centre, University of Helsinki, Helsinki, Finland, [2] Current address: Department of Plant Sciences, University of Saskatchewan, Saskatoon, Canada and [3] International Center for Agricultural Research in the Dry Areas (ICARDA), Rabat, Morocco

*Corresponding author: H. Khazaei, ORCID ID: 0000-0002-5202-8764, contact: hamid.khazaei@gmail.com, call @ +1 (306) 361-1167

Running title: **Epicuticular wax diversity in faba bean**

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Abstract

Among grain legumes, faba bean is reputed to be relatively sensitive to drought stress. Epicuticular wax (ECW) quantity is considered as an important drought adaptation strategy in plant species. This study aimed to define variation in leaf ECW concentration as a drought-adaptive trait in 197 faba bean accessions under well watered conditions. The relationship between ECW and stomatal characteristics was also investigated. Highly significant differences were found in the ECW concentration, which ranged from 0.680 to 2.104 mg/dm^2. No relationships were found between ECW and any measure of stomatal morphology and function. This study provides evidence of wide variation in ECW in faba bean germplasm, which is independent of stomatal characteristics and leaf water content. This variation may allow the genetic improvement of ECW as a drought-adaptive character in faba bean breeding programs aiming at economical use of water.

Keywords: epicuticular wax, genetic diversity, faba bean, drought, stomata

Introduction

Faba bean is an excellent source of protein for human food and animal feed, and it is suitable for environmentally sustainable agriculture due to its generous ecological services. Nevertheless, faba bean is reputed to be relatively sensitive to drought (McDonald and Paulsen, 1997; Daryanto *et al*., 2015). Various morpho-physiological and biochemical characters confer drought adaptation in plants. Several studies have demonstrated faba bean drought-related morpho-physiological traits in above-ground (*e.g*., Darwish and Fahmy, 1997; Khan *et al*., 2010) and below-ground characteristics (*e.g*., Belachew *et al*., 2018). The waxes on plant cuticles form a protective cover against biotic and abiotic stresses (Jenks and Ashworth, 1999; Xue *et al*., 2017). The effectiveness of stomatal control over transpiration efficiency increases if non-stomatal transpiration pathways are restricted. An important non-stomatal water vapour pathway is the leaf cuticle.

Accumulation of epicuticular wax (ECW) is considered an important drought avoidance strategy in many plant species. In pea (*Pisum sativum* L.), a greater amount of epicuticular wax was correlated with a higher harvest index under drought conditions (Sánchez *et al*., 2001). Drought stress enhanced the deposition of cuticular waxes and subsequently improved drought adaptation in alfalfa (*Medicago sativa* L., Zhang *et al*., 2005) and soybean (*Glycine max* (L.) Merr., Kim *et al*., 2007). Variation in the wax quantity has been demonstrated in several crop species (reviewed in Sharma *et al*., 2018) but not yet in faba bean. Thus, this study aimed to explore variation in leaf ECW concentration as a drought adaptation character in a relatively large faba bean germplasm collection.

Experimental

A set of 197 faba bean accessions from the ICARDA collection was used in this study (Table S1).

Faba bean germplasm was grown in 2 L plastic pots containing a mixture of sand and peat (3:1 *v*/*v*) in a climate-controlled glasshouse at the Department of Agricultural Sciences, University of Helsinki, as reported previously (Khazaei *et al*., 2013). The experimental design was a randomized complete block with four replicates. Throughout the experiment, soil moisture level was maintained at field capacity. Photoperiod was adjusted to 14 h/10 h (light/dark), and the temperature was maintained at 21°C day/15°C night. Minimum daytime PPFD was set to 300 μmol m^{-2} s^{-1} at the canopy level. Relative humidity was kept at 60%.

Eight weeks after sowing, the following measurements were taken on the youngest, fully expanded leaves. Leaflet area was measured using a LI-6200 leaf area meter (LI-COR Inc., Lincoln, NE). Stomatal density and area were measured following the impression method (Wang and Clarke 1993). Gas exchange traits were measured using a LI-6400 portable photosynthesis system. Canopy temperature was measured using a FLUKE® 574 infrared gun (Fluke Corp., Everett, WA). Leaf relative water content was measured as described by Barrs and Weatherley (1962).

The amount of ECW was determined by a colorimetric wax quantification method developed by Ebercon *et al*. (1977), based on the colour change produced by the reaction of ECW with $K_2Cr_2O_7$. The reagent was prepared by mixing 20 g of powdered potassium bichromate with 40 ml of deionized water. The slurry was mixed with 1 L of concentrated sulfuric acid and heated at 80°C until a clear solution was obtained. To extract wax, 10 fresh leaf discs having a total area of 40 cm^2 (both surfaces) were punched from the leaves. Samples were immersed in 17 ml of chloroform for 15 s. The chloroform-ECW solution was filtered through a fine filter paper, then evaporated on a boiling water bath until the odour of chloroform was gone. Next, 5 ml of $K_2Cr_2O_7$ reagent was added into glass test tubes and heated at 100°C in a water bath for 30 min. After cooling, 12 ml of deionized water was added, and the colour was allowed to develop for at least 3 h. The optical densities of the samples (two readings per replicate) were measured at 590 nm with a PharmaSpec UV-1700 UV–Visible Spectrophotometer (PharmaSpec, Shimadzu, Germany).

For reference purposes, faba bean wax was extracted from a large amount of field-grown leaves using chloroform. A standard curve was prepared by following a serial dilution method from ECW extracted from PEG-3000 (polyethylene glycol), Carnauba wax, and faba bean wax. The linear standard curve equation was used to determine the wax concentration of samples.

The R statistical package (R Development Core Team, 2016) was used for all data analysis. The means of studied traits are given in Table S2 and S3.

Results and Discussion

The standard curves for all three tested waxes were linear throughout the concentrations (Fig. S1). PEG-3000 was curved slightly downward and Carnauba slightly upward while faba bean wax was straight. The faba bean wax was used as the standard for ECW measurements.

The 197 accessions differed for ECW ($P < 0.001$). ECW ranged from 0.680 mg/dm^2 to 2.104 mg/dm^2 (Fig. 1; Table S2), with a mean ± SD of 1.288 ± 0.263 mg/dm^2. ECW showed no significant correlations with stomatal morphology, gas exchange traits and relative water content, and only a weak positive association with leaflet area ($P < 0.090$) (Table 1). The lack of associations between ECW and other leaf traits related to drought adaptation, particularly stomatal characteristics under non-stress conditions, indicates that faba bean might use both rapid stomatal closure and increased wax accumulation to reduce water loss when adapting to drought. In stress conditions, however, ECW was associated with the gas exchange traits in Jatropha (Figueiredo *et al*., 2012) and wheat (Huggins *et al*., 2018). It should be noted that leaf mesophyll conductance, chloroplast density, and cell wall thickness also regulate water balance in plants (Ouyang *et al*., 2017).

Here we employed a high-throughput screening colorimetric method for screening a large number of faba bean accessions for ECW. To our knowledge, no previous study has shown the variability in ECW in faba bean. Accessions IG 12132, IG 12260, IG 11989, IG 137686, IG 72375, IG 112096 and IG 74554 had the highest ECW concentrations among accessions. These accessions might be used in genetic improvement of ECW as a component of drought adaptation in faba bean breeding programs.

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Figure legends

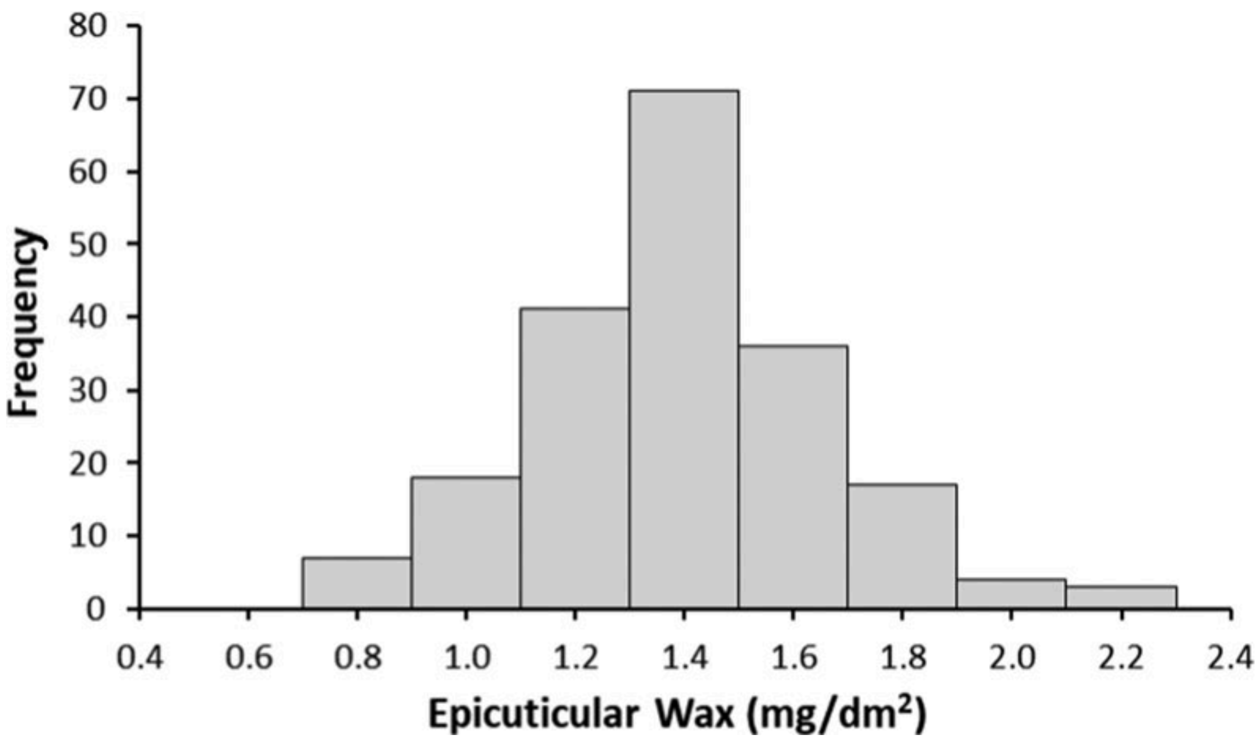

Fig. 1. Frequency distribution of leaf epicuticular wax concentration in 197 faba bean accessions (Skewness= 0.316, Kurtosis= 0.474). $LSD_{0.05}$ (least significant differences) = 0.059.

Table 1. Pearson correlation coefficients of epicuticular wax content (ECW) with morpho-physiological traits among 197 faba bean accessions.

	LA	**SD**	**SA**[a]	**g_s**	**E**	**TE**[b]	**CT**	**RWC**
Correlation with ECW	0.121	0.099	-0.088	0.030	0.042	0.000	-0.040	0.029

[a] Stomatal length × stomatal width.

[b] Transpiration efficiency was calculated as photosynthetic rate divided by the stomatal conductance.

LA, leaflet area; *SD*, stomatal density; *SA*, stomatal area; g_s, stomatal conductance; *E*, transpiration rate; *TE*, transpiration efficiency; *CT*, canopy temperature; *RWC*, relative water content.