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# Obtaining new varieties of *Vicia faba* L.: characterization of a population of inbred lines

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*To my Families,*
*the Biological one*
*and the Chosen one.*

*To whom made me fall*
*in love with this work.*

*"You can do anything if you have enthusiasm. Enthusiasm is the yeast that makes your hopes rise to the stars. Enthusiasm is the spark in your eye, the swing in your gait, the grip of your hand, the irresistible surge of your will and your energy to execute your ideas. Enthusiasts are fighters, they have fortitude, they have strong qualities. Enthusiasm is at the bottom of all progress. With it there is accomplishment. Without it there are only alibis."*

***Henry Ford***

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# Abstract

Faba bean is a high-protein legume crop that is widely used in human feeding and livestock feed.

Despite its value, the global area under faba bean cultivation has decreased over the last five decades. Not only its production is constrained by several biotic factors and abiotic stresses, but also the presence of anti-nutritional factors reduces the use of this legume.

We have indentified 51 inbreed lines with a low tannin content, that have been phenotypically characterized by examining 22 traits, as provided by the UPOV (International Union for the Protection of New Variety of Plant procedure) guidelines for faba bean. The similarity analysis shows that even if some interesting traits (low tannin, absence of anti-nutritional factors, high level of self-fertility) have been fixed, a great variability is present in the population, fully exploitable to obtain new varieties.

A performance assessment on the field and a comparison between the lines through the experimental scheme of Augmented Block Design, have been performed to identify the best genotypes for the development of new synthetic varieties. Seven new synthetic varieties were created, with the characteristics of the triple white (TW), that have already been tested in the field for two years.

Furthermore, a selection of synthetic lines and of best inbred lines of the population were tested, in controlled conditions, for resistance to two major diseases that affect the faba bean: Rust and Chocolate spot. Three inbred lines, IS28, IS48 (both TW) and IS89 with a good response to both diseases have been identified. The best of the Synthetic lines tested it is IS-Sint4.

The work has allowed us to identify and characterize some inbred lines and synthetic, which will be multiplied, evaluated and developed in order to create a new commercial variety that could be a viable alternative to existing varieties, with a focus on yield stability and low content of anti-nutritional factors.

# Summary

# *Introduction*

## Motivations

The main objective of this research, developed within a collaboration between the Polytechnic University of Marche and the seed company ISEA, is the characterization of a breeding population of *Vicia faba* L., for: agronomic characteristics; qualitative traits; characters related to resistances to biotic stresses. The final aim of the work is to obtain new marketable material that has good performances and the triple-white (white flower, white seed coat and white hilum) characters, associated with the absence of anti-nutritional factors, which, along with the unstable yield and susceptibility to pathogens, are among the main problems that reduce the uses of this species.

## Structure of the thesis

The thesis is structured in three chapters.

In the first chapter, it is shown a description of the specie and an overview of the state of the art about main breeding objectives for the Faba bean

In the second chapter is reported a study on 83 lines of faba bean, native to the Mediterranean basin, that were characterized for agronomic and qualitative traits, in order to evaluate the potentialities of the population

for the development of new marketable varieties of *Vicia faba* L. for various commercial classes. The aim of the work described in the second part of the chapter 2, was to evaluate seven synthetic varieties created from inbred lines with the characteristics of the triple white, with the final aim to obtain new marketable varieties with the characteristics of the triple white, exploiting the advantage agronomic given by heterosis.

In the third chapter is described the characterization, under controlled conditions, of a selection of inbred and synthetic lines for their response to Rust and Chocolate spot, focusing on the identification of potential sources of resistance.

CHAPTER 1

# The Genetic Improvement of *Vicia faba* L.

## 1.1 *Vicia faba* L.

Faba bean (*Vicia faba* L.) is a legume of the *Fabaceae* family, genus *Viciae* (Pignatti, 1982), and a very ancient culture. Studies claim that probably the species was domesticated during the Neolithic (Shultze-Motel, 1972), both in the Middle East (Cubero, 1974), and in Central Asia (Ladizinski, 1975). The name of the species derives from the greek φάγέώ, that means eating, etymology which also demonstrates its use as a food among the ancient Greeks and Romans (Muratova, 1931; Hopf, 1937): at that time was the most important legume crops for both food and feed (Bozzini and Chiaretti, 1997).

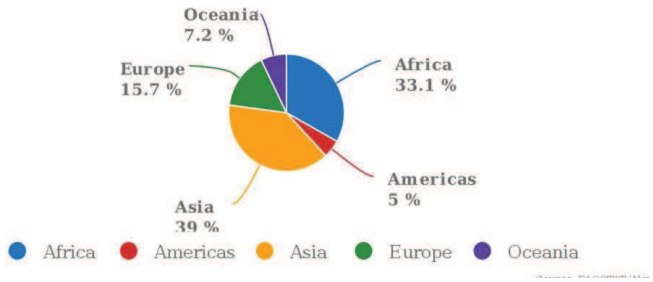


**Fig. 1.1 Production share of Broad beans, horse beans, dry by region (FAO, 2016)**

The broad bean is grown in the world on an area of approximately 2.37 x $10^6$ ha. The world's top producing country is China, followed by

Ethiopia and Australia. Italy is the eleventh producing country in the world (FAO, 2016).

Despite its popularity since its domestication in Europe and in the world, the broad bean consumption has declined significantly during the twentieth century (Duc, 1997). This is an inevitable consequence of the replacement, in agriculture, of animal work with machinery (the bean was the main ingredient supply of oxen, horses and mules. Not surprisingly, one of the *Vicia faba* names is "horse bean"), and also because there was no plant breeding efforts to obtain new varieties, suitable to modern agriculture.

The species has an intrinsic ability to adapt to diverse climates, but its low and unstable yields, the susceptibility to biotic and abiotic stress, and the presence of anti-nutritional factors, hamper its competitiveness as a crop (Duc *et al*., 2015).

The uses of faba bean are many and varied. Since antiquity, the fava beans were stored dried, to then be rehydrated and used for human consumption. Recently, seeds of varieties of dried broad bean with no tannins, such as dried seeds of other legumes such as lentils, peas, chickpeas, beans, are rehydrated and canned. The fresh green seeds of medium to large size (*Vicia faba* ssp. *major*) are used as a vegetable, consumed directly or cooked. Today we also find on the market, frozen fresh green seeds, as well as other common legumes (like beans or peas), ready to be defrosted and then cooked before use (Bozzini and

Chiaretti, 1997; Duke, 1983). The field bean, *Vicia faba* spp. *minor* (such as pea protein), is an important agricultural species for crop rotations; it is a crop suitable for clayey and heavy soils (Jensen *et al.*, 2010).

Within the species, three botanical varieties are distinguishable on the basis of seed size:

- *Vicia faba major*: broad bean, which produces flattened and large seeds (100 seeds weigh from 100 to 250 g). Large-seeded cultivars are widely preferred for food use, whether as a fresh green vegetable or dry;

- *Vicia faba minor*: field beans, whose seeds are rounded and relatively small (100 seeds weigh less than 70 g) and are used for sowing grass meadows and green manure and as concentrates for livestock feed. Small-seeded cultivars are preferred for the specialist market for feeding pigeons (i.e. the UK cultivar "Maris Bead") and easy drying after harvest in high latitudes such as the Nordic region and the Canadian province of Saskatchewan;

- *Vicia faba equina*: horse bean, with flattened seeds of medium size (100 seeds weigh from 70 to 100 g), which are used for livestock feed and, today, even as human food, like fresh chopped canned or frozen. The most widely grown cultivars, mostly used for animal feed in European cultures, and for use as dry pulses in West Asia and North Africa, have medium-sized seeds.

Seed size is important for meeting market and farmer needs, although it is a continuously variable property. (Baldoni *et al.* 1981; Bonciarelli, 1998; Duc *et al*., 2015).

Faba bean provides valuable ecological and environmental services in sustainable agriculture, diversity in cropping systems, and host numerous associated organisms including pollinating insects. The capacity of this species to establish symbiosis with specific rhizobia bacteria results in biological nitrogen fixation which reduces the input of fertilizers in arable lands. (Duc *et al*., 2015).

## 1.2. The variability of *Vicia faba* L.

The faba bean genome (2n = 2x = 12), is one of the largest among legumes (1C = 13.33 pg ≈ 13.000 Mbp, Bennett and Smith, 1976 and Johnston *et al*., 1999).

Among the subspecies and the botanical varieties ascribed to *V. faba*, there are no sterility barriers. The reproductive system of the species is partially allogamous. Cross-pollination is generally mediated by hymenopterans, such as bees and bumblebees (Baldoni *et al.,* 1981; Suso *et al.,* 1996).

Various levels of tolerance to winter conditions helped to distinguish between spring-sown and autumn-sown cultivars. They differ in their

level of frost hardiness, vernalisation requirement, day-length response and flowering time and fungal disease resistance. Conversely, the boundary between the two cultivar types is not always clear cut and some spring cultivars of Northern Europe may be grown in winter in some Mediterranean zones.

Being a very old crop, *V. faba* has an enormous genetic variability. It can be useful for the improvement of the species; adapted to a wide range of latitudes and altitudes. It has been farmed in winter and spring, with differences in the uses, i.e. for food and feed (Maxted *et al*., 2000). Ought to technical difficulties in making interspecific crossings or producing transgenic lines of *V. faba*, breeders can only count on the great variability existing in nature or that induced by mutagenesis (Duc, 2010).

Being the wild progenitor unknown, all genetic diversity available is contained in *ex situ* collections and local populations maintained by farmers in traditional agricultures. Faba bean germplasm of over 38,000 accessions is conserved worldwide in, at least, 43 national gene banks (table 1.1), as well as at the International Center for Agricultural Research in Dry Areas (ICARDA). ICARDA safeguards the largest collection in the world, with materials from 71 countries with a high percentage of unique accessions (Duc *et al*., 2015). The adequate *ex situ* conservation of faba bean collections is limited by the outcrossing floral biology of the species and its low multiplication rate (Suso *et al*. 2011).

**Tab. 1.1: Major *Vicia faba* ex situ collections worldwide in 2014 (Duc *et al*., 2015).**

ICARDA International Institute for Agricultural Research in Dry Areas, CAAS Chinese Academy of Agricultural Sciences, IPK Institute of Plant Genetics and Crop Plant Research, INRA Institut national de la recherche agronomique, CNR Consiglio Nazionale delle Ricerche, IOPG Institute of Packaging, Ghana, PGRC Plant Genetic Resources Centre, IFAPA Instituto de Investigación y Formación Agraria y Pesquera. Anterior, PBAI Plant Breeding and Acclimatization Institute, INRB National Institute of Biological Resources, USDA US Department of Agriculture, DLO Dienst Landbouwkundig Onderzoek, IIPGR Institute for Introduction and Plant Genetic Resources

| Country | Institute/city | Number of accessions |
|---|---|---|
| Syria | ICARDA/Aleppo | 10,045 |
| China | CAAS/Beijing | 5900 |
| Australia | Australian Grain Gene Bank/Victoria | 2445 |
| Germany | Gene Bank IPK/Gatersleben | 1920 |
| France | INRA/Dijon | 1900 |
| Russia | VIR/St Petersburg | 1881 |
| Italy | Genebank/Bari | 1876 |
| Morocco | INRA/Rabat | 1715 |
| Spain | CNR/Madrid | 1622 |
| Poland | IOPG-PA/Poznan | 1258 |
| Ethiopia | PGRC/Addis Ababa | 1118 |
| Spain | IFAPA/Cordoba | 1091 |
| Poland | PBAI/Radzikow | 856 |
| Portugal | INRB—IP/ Oeiras | 788 |
| USA | USDA/Pullman | 750 |
| The Netherlands | DLO/Wageningen | 726 |
| Bulgaria | IIPGR/Sadovo | 692 |
| **Total: world** | **More than 43 known collection** | **38,360 accessions** |

At the end of the twentieth century, in European countries and also at ICARDA (Syria) have been made large investments in the improvement of *Vicia faba*. Mainly in the study of the discovery of genetic variability and in breeding activity for traits of agronomic interest and particularly, in tolerance to several biotic and abiotic stresses (Bond and Poulsen 1983; Duc, 1997). A large genetic variability has been identified in terms of floral biology. Cytoplasmic and nuclear determinisms of male sterility, various flower architectures or colours and various levels of autofertility and attractivity for pollinating insects were described. This could help to manipulate the levels of outcrossing (Link *et al.*, 1994; Duc, 1997), which it is a major problem for the conservation of accessions in the collections.

Wide phenotypic variability was observed for the pigmentation of the stem; the shape and size of the leaves; the tendency to lodging; the colouring, in the flowers, of the wings and the banner; the angle, form, villosity, colouring and distribution of the pods; seed coat and hilum colour; shape and longitudinal section of the seeds. Different combination models of the traits listed above were found for eight pure lines of local origin; in the valley of the Nile and Ethiopia, it was found the trait of low pigmentation of the stem; in Europe, the high frequency of small leaves, against the low frequency observed in the Nile valley; pods erected in the Indian subcontinent, pendants in South Europe, dark in Ethiopia (Robertson *et al*., 1991).

A set of about 5000 faba bean accessions from the National Genebank of China was randomly sampled and analysed for plant height, which averaged 78 cm (Zong *et al*., 2006). Referring to the traits of yield component, the number of effective pods ranged from 1.1 to 93.7. The number of mature seeds per pod is from 0.8 to 6.1. The 100-seed weight of dry grain ranged from 6 to 240 g and the dry grain yield per plant from 1.2 to 127.0 g. The mean dry pod length was 6.5 cm (range 1.2–18.8 cm) and the width 1.6 cm (range 0.7–3.5 cm; Zong *et al*., 2006).
Flower colour is subject to oligogenic determination of major traits. A black dot is often present on the wing petals, and the flowers can be pure white or with diffuse pigments on all petals (purple or dark brown; Picard, 1976). Recessive alleles at either of two genes (zt-1 and zt-2) can determine the pure white flower trait and have a pleiotropic effect on the seed coat composition determining the absence of tannins. The absence of tannins in seeds, improving the protein digestibility in monogastric animals resulted in a "zero-tannin" group of cultivars while low contents of vicine and convicine in seeds, improving feed value in poultry and reducing favism risks in humans, define a "low vicine–convicine" group (Crépon *et al*., 2010). Faba bean seeds display large genetic variation in seed coat colour and pattern (spotted, marbled), hilum colour and cotyledon colour, traits that have been described with a strong relationship with tannin content (Picard 1976; Duc *et al*., 1999).

Genetic resources are continuously under evaluation for resistance or tolerance mechanisms against biotic and abiotic stresses (Khan *et al*., 2010; Duc *et al*., 2011). Ascochyta blight, chocolate spot, rust, downy mildew, Fusarium spp., broomrapes, nematodes, aphids, sitona weevil and bruchids are the major parasites or pests so far reported for which sources of genetic resistance are needed.

Biotic stresses may be very different in terms of variability and extent of damage, according to geographic zones and date of sowing. The wide dispersion of faba bean has resulted in regional adaptations to abiotic stresses and variation in disease resistances (Duc *et al*., 2015).

Resistance to chocolate spot has been found in Ecuador, tolerance to heat stress in Bangladesh, frost tolerance in winter types from Europe, drought tolerance in the Mediterranean region, plus genetic resistances to Ascochyta blight, nematodes, bean yellow mosaic virus (BYMV) and bean leaf roll virus (BLRV) and broomrapes. Genetic resistances of faba bean to its major fungal diseases in temperate regions: chocolate spot, Ascochyta blight, rust, downy mildew (Sillero *et al*., 2010) and to its important parasitic plant Orobanche (Fernandez-Aparicio *et al*., 2012; Rubiales and Fernández-Aparicio, 2012) have been identified.

Zong *et al*. (2006) analysed, for the nutritional composition of the seed, 1828 faba bean accessions from the National Genebank of China. On total seed dry matter, the main components were: crude protein, ranged

17.6–34.5 %; total starch, ranged 33.2–53.4 %; while amylose of starch, ranged 6.0–27.9 %; lipid, ranged 0.52–2.80 %.

A similar range of variations was described for these main seed constituents in a European seed collection (Duc *et al*., 1999), where mean seed coat proportion ranged 11.0–14.8 %, (the zero tannin cultivars being 2 % lower than tannin-containing ones), mean neutral detergent fibre ranged 13.4–21.7 %. In a set of 72 accessions, amylose content ranged 17–29 % of starch, with a significant tendency for lower content in larger-seeded accessions. White-flowered genotypes were tannin-free; coloured-flower genotypes contained 6–10 g/kg of condensed tannins, concentrated in the seed coat.

An allele (*vc*-) of the VC gene has been discovered (Duc *et al*., 1989) which reduces by 10–20 times the content of vicine and convicine in the seed. Vicine–convicine are two glucosides, concentrated in cotyledons, responsible for favism risk in humans and lower production performances in chickens or laying hens (Arese and De Flora 1990; Crépon *et al*. 2010). The content in conventional genotypes is 6–12 g/kg of seed dry matter, and it is about 0.5 g/kg in *vc*- homozygotes (Duc *at al*., 2015).

The main goal of national breeding programs for this species is to obtain higher-yielding new cultivars, with improved combinations of disease resistances and quality. So, the importance of ex -situ gene banks in collecting and preserving local landraces, with their associated ranges

of adaptations to respective crop environments, is crucial to obtain new varieties for the different national markets (Maxted *et al*., 2000, Duc *et al*., 2015).

## 1.3. Genetic improvement of *Vicia faba* L.

Historically, the primary objective of breeders is to get more and better varieties, to be introduced on the market.

To get a new variety can take years, especially if the genetic material of departure is not already improved material. In general, the choice of a breeding method, you must consider several variables:

1. *The reproductive system,* including floral biology, frequency of natural fertilization and the trait to be improved.

2. *The genetics of the trait.* Traits can be mono-, oligo- or polygenic and even cytoplasmic (i.e. CMS male sterility; Picard *et al*., 1982; Duc, 1997).

3. *Environment-genotype interactions*. Because of the high cost of developing a new cultivar, breeders seek to create plastic variety, of wide adaptation to different environments. However, given the variability of the world's environment, it is important to keep in mind the characteristics of the environment where a new variety will be cultivated, where a plastic variety could not

represent an advantage over a narrowly adapted one. It also happens in special environments, where the susceptibility and adaptation to abiotic stress is crucial (such as acidic, alkaline or saline soils, soils rich in heavy or toxic ions, dry or cold regions, etc.)

4. *The final product*. Depending on the characteristics and value, in economic terms, the species, the breeder can choose whether to get an open pollinated population, a synthetic or a hybrid variety, a pure line or a multiline cultivar. (Ranalli and Cubero, 1997)

*Vicia faba* L. is a very peculiar species from the point of view of breeding choices, given its partially allogamous nature. The range of natural outcrossing of faba bean is between 2% and 84% (Bond and Poulsen, 1983) depending on geographical location and the activity of pollinating insects at the time of flowering.

It has been attempted to obtain closed flower mutants to force autogamy and make possible the breeding for autofertility (Knudsen and Poulsen, 1983). The mutants induced exhibited flowers that were not tightly closed and outcrossing rates ranging from 5 to 23%; consequently, this crop is subjected to breeding methods of the partially outcrossed populations and the selection is aimed at developing synthetic and hybrid varieties (Ranalli and Cubero, 1997).

Because of heterosis, synthetic and hybrid faba bean cultivars perform better and have greater yield stability than inbred cultivars (Bond, 1982; Bond, 1986; Scheybal, 1988). Yield increases of 20 to 50 per cent have been reported for F1 hybrids (Ranalli and Cubero, 1997).

In the past, attempts were made to get forced Allogamy, by male sterility. Breeding hybrid faba bean cultivars is not yet feasible however, due to instability of the male sterility found in the species (Bond, 1987; Bond, 1989). Many faba bean lines with the character of male sterility have been found, both spontaneous (Bond *et al*., 1964), both induced by mutations (Duc, 1984). The nuclear male sterility type (NMS) has been excluded from breeding programs, because they were not found good morphological markers that allow the exclusion of fertile plants among the male sterile plants. Cytoplasmic male sterility (CMS), which is much better suited to breeding programs, is a possible way of NMS, but experienced instability (Picard *et al*., 1982; Duc, 1997).

The instability is due to the fact that the CMS is carried by mitochondrial RNA, virus-like, which reproduce in number and in ways not always equal, and there exists a number of RNA copies limit threshold below which CMS does not work. It was put to an ELISA test point (Dulieu *et al*., 1988) for selecting the number of RNA present. In any case these instability problems make the choice of the breeding for obtaining of hybrid faba bean, not viable and economically not advantageous.

A good breeding strategy for faba bean includes various steps:

1) *Recurrent selection:* the breeder starts from a population and begin to improve it with recurrent selection. This selection could be done in nurseries using natural crossing to provide recombination;

2) *Development of inbred lines selected by pedigree method:* long but effective method. You can accelerate using greenhouses or fields located in different parts of the world. Single-seed, or single-pod descent, has been advocated but has not proved very popular among field bean breeders. A more useful trend in practice has been to evaluate partially inbred lines (at early generation, F1 to F3) and their performance related to more advanced inbred lines (i.e. F15, F16): evaluation of lines early in the course of their inbreeding at least ensures that further, expensive, inbreeding is carried out within only the more promising families (Bond, 1987);

3) At this point one can proceed with the development of:

    a) *Synthetic varieties:* an easy way to obtain an advanced variety with a yield that is more than the average of their inbred components, also considering the fact that inbreeds are less stable than composites;

    b) Hybrids: heterosis in faba bean is well documented, but there are yet no F1 hybrid varieties giving it full commercial exploitation (Ranalli and Cubero, 1997). No investments have

been made in this regard, because the economic value of this species is not comparable to other, such as corn or wheat.

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CHAPTER 2

# Phenotypic characterization of a breeding population

## 2.1 Introduction: Breeding goals for faba bean

The instability of the yield of *V. faba* is unfortunately well-known, indeed, to increase and stabilize the yield is the major objective for the breeders. In most of the cases, this production instability is evident in advanced generations of inbreeding (F6 and F7), and in trials located in different places; hybrids instead appear to be more stable, and make better use of the land (Duc, 1997).

### 2.1.1. Improvement of seed characteristics

*Dimensions*

Among the cultivated crops in most temperate farming systems, faba bean has the highest individual seed size. 100 seeds weight (100SW) can easily vary between 30 and 200 g (China and Japan) within breeding material, and in released cultivars for Western Europe it ranges from 45 to 65 g. The size of the seed has a high heritability, with a considerable additional genetic component, and in general with positive correlation with the yield, but the conditions during ripening

determine whether the expression of the genetic seed size is at the higher end (optimal conditions) or the lower end (stress, drought): the variation within one cultivar can differ up to 150 g depending on the conditions of the production site.

The large seed of faba bean offers a large surface area that is exposed to mechanical impact, and a large diameter that requires adequate time for moisture to move from the interior of the seed to the seed coat where it is released. The cost of the seed is very high for farmers, but it can be reduced by using smaller seeds. So, the choices of breeding are taking the direction of more and more small seeds (Duc, 1997; Duc *et al*., 2015).

*Composition and quality*

When faba bean is produced for human consumption, consumers set the standards according to their preferences, the size, shape, colour and resistance to cooking, depending on usage.

Two glucosides, *vicine and convicine*, anti-nutritional compounds found in many varieties of faba bean, cause Favism, an haemolytic anaemia (that can lead to death) due to the ingestion of faba beans or after inhalation of pollen from the plant, by people with glucose-6-phosphate dehydrogenase (G6PD) deficient erythrocytes. The elimination of vicine and convicine from *V. faba* is the goal of many researchers.

Maintaining a high *protein content* is very important, especially for the feeding of poultry. The protein content of the bean, varies between genotype and genotype, in a range that goes from 27 to 34% in the dry seed, of which 80% are globulins. A comparison with the soybeans, the species *Vicia* seeds are rich in lysine but low in sulphur amino acids (Sjödin *et al*., 1981) and tryptophan. Observing data collected during the EU-CAMAR program (1990-1995), during which were taken into account the combined effects of the genotypes with the environment, you may notice that in the high-protein genotypes can get higher content methionine and cysteine. The fraction of albumin, includes protease inhibitors and lectins at similar concentrations to those of spring peas, which have a deleterious effect uncertain for nutrition (Bond *et al*, 1993).

The *tannins* are considered the main anti-nutritional factor of faba bean, reducing its digestibility. Recessive alleles at either of two genes (*zt-1* and *zt-2*) can determine the pure white flower trait and have a pleiotropic effect on the seed coat composition determining the absence of tannins (Picard, 1976; Duc *et al*. 2015). The *zt-1* gene has been found in many European cultivars with white flower, while *zt-2* is less common, but according to the data obtained by the ÉCLAIR-PEA program, appears to lead a higher energetic value for the feeding of poultry, compared to *zt-1* (Duc, 1997).

*Vicine and convicine*, as well as being the cause of the manifestation of Favism in humans, lead to poor production performance of laying hens. Were discovered individuals natural or induced mutants, with an almost zero content of vicine-convicine and the same phenotype of those-containing glucosides (Duc *et al*., 1989; Ramsay and Griffiths, 1993). This hereditary trait is monogenic, then simple to select using a simple spectrophotometry (Sixdenier *et al*., 1996). It was also observed that vicine-convicine content decreases due to the presence of the *vc*- allele, linked to the colourless hilum gene (Duc *et al*. 1989, Duc, 1997), which can then speed up the work of breeders.

The absence of tannins in seeds, improving the protein digestibility in monogastric animals resulted in a "zero-tannin" group of cultivars while low contents of vicine and convicine in seeds, improving feed value in poultry and reducing favism risks in humans, define a "low vicine–convicine" group. The combination of both traits has received the generic name "fevita" (Crépon *et al*. 2010; Duc *et al*., 2015).

### 2.1.2. Improvement of morphological and agronomic characteristics

*Early flowering*

Faba bean is known for its susceptibility to drought at the time of flowering. To escape from the stress due to drought and allow the cultivation of field beans in the driest areas, breeders have worked

especially on the precocity. The time of flowering is usually a high heritability trait, and can have a simple genetic basis, as in OPTICA cultivars, where a single dominant gene was identified for early flowering (Le Guen and Duc, 1992).

*Plant architecture*

Spontaneous genetic variation and induced mutations have been widely exploited to reduce entrapment and abortion of the reproductive organs (Bond, 1987). The *ti* gene induces a determined development of the stem, with the terminal inflorescence after 3-7 floral nodes; this gene has been used in many improvement programs, but there have never been significant results in production (Sjödin, 1971; Silim and Saxena, 1992; Stützel and Aufhammer, 1992; Malhotra *et al*., 1995).

Frauen and Sass (1989), identified the character of lignification of the stem, which has a simple monogenic inheritance. This gene confers the reduction of the growth of the stem without damaging the growth of seeds (i.e. cv. Tina) (Duc, 1997).

Most breeders agree that a high number of pods per flowering node is a positive feature. In general, the architecture attempts modification of the plant, are directed to build a plant that has: greater compactness, reproductive synchrony in development, and reduced vegetative growth.

### 2.1.3. Improvement for abiotic stresses

Faba bean, like all crops, has optimum temperatures, water and mineral requirements for growth, and conditions outside these ranges cause stress.

The plant height, the number of stems and pods per plant, harvest index, the weight of 100 seeds, date of flowering and early maturation, are the most important characters in yield improvement, related positively or negatively to this (Loss and Siddique, 1997). Several studies have shown that the heritability of production traits (in particular of the number of stems and of pods per plant, biomass yield and number of seeds) decreases greatly with the worst environmental conditions of cultivation (Abdelmula, 1999; Link, 1999; Toker, 2004). Climate change is likely to increase the frequency of heat and water deficit stresses, but may also affect the exposure to other stresses. Recovery from a transient stress is as important as survival, but it is less often examined in experiments (Duc *et al*., 2015).

The tolerance to *salinity and drought*, is very important in some areas of the Mediterranean. Some physiologists and botanists have also found a relationship between this tolerance and the symbiosis with *Rhizobium* (Guerin *et al*., 1990; Cordovilla *et al*., 1994).

Drought responses are usually characterized as: escape, avoidance and tolerance. To escape the stress due to drought and allow the cultivation of field beans in the driest areas, breeders have worked especially on

the precocity. To provide tolerance, instead, they worked on improved water metabolism, through better root systems, and stomatal closure, that represents avoidance and protection of cell metabolism through an osmoprotective substance such as glycine, betaine or proline (Duc *et al*., 2015).

Escape is not always sufficient to escape drought stress, i.e. against transient, mid-season water deficit. There is direct evidence for wide variation in stomatal traits, and there is indirect evidence from the same experiments for variation in the ability of roots to obtain water (Khazaei *et al*. 2013).

Canopy temperature depression is an effective, rapid and economical way to determine the water status of a faba bean plant (Khan *et al*. 2010; Khazaei *et al*. 2013). Osmotic adjustment through the synthesis of protective substances has not been identified in faba bean (Duc *et al*., 2015).

The tolerance to *cold* is a very important component for the resistance to cold winters, and for this character is known a large genetic variability. Faba bean is well adapted to cool temperatures and seldom shows damage from temperatures down to - 6 °C, although flowers are not tolerant of frost (Link *et al*. 2010). The trait of the cold tolerance showed an association with the gene of the late flowering, a feature not positive. The breeders are working to break this bond (Duc, 1997). One

of the biggest sources of precocity was discovered tied to the terminal inflorescence gene, the gene *ti* (Stoddard and Hämäläinen, 2011).

In continental and oceanic regions, the autumn-sown faba beans have to withstand very hard frosts, often in repeated freeze–thaw cycles, and in some regions prolonged snow cover as well. The assemblage of traits required for winter tolerance is complex (Link *et al*. 2010) and has seen some attention to individual components such as frost tolerance (Arbaoui and Link, 2008; Arbaoui *et al*., 2008) and tolerance to snow cover (Fukuta and Yukawa 1998).

Early maturity is a desired attribute in other zones besides Mediterranean climates. Faba bean often matures after the small-grained cereals, whether spring sown in boreal climates or autumn sown in oceanic climates. There is a fundamental conflict between the aims of earliness and high yield, because the more radiation that a crop can intercept through a longer growing period, the greater its potential biomass production (Duc *et al*., 2015).

### 2.1.4. Improvement for biotic stresses

Faba bean is subject to a range of diseases, parasites and pests, and resistance breeding is an important issue with priorities differing from region to region (Duc *et al*., 2015).

The resistances to pathogenic fungi, as part of the practice necessaries for integrated disease management, reduce the need for application of fungicides but provide incomplete resistance. So, it is important that

those resistances are combined with good practices such as: crop rotation (and choice of the species); sanitary control of certified seeds (i.e. the case of *Ascochyta fabae*); reduction of relative humidity in the canopy (by: lower sowing rate, good soil drainage, choice of plant architectures; prevention of lodging); targeted period of sowing and plant cycle; prevention of nutrient deficiencies, frost damage and weed infestations (Stoddard *et al*., 2010).
In Chapter 3, a more extensive discussion about the improvement for resistance to biotic stress.

## 2.2 Objective of the work

This study, developed in a collaboration between the Polytechnic University of Marche and the seed company ISEA, aims to characterize phenotypically and agronomically, 83 lines of faba bean, in order to evaluate the potentialities for the development of new marketable varieties with the characteristics of the triple white (white flower, white seed coat and white flower), for various commercial classes, of *Vicia faba* L. The lines originate from a ENEA breeding program (Bozzini and Chiaretti, 1997; 1999)., then have been selected and developed by ISEA (in collaboration with ENEA). We also evaluated, at agronomic level, 7 synthetic varieties created from inbred lines with the characteristics of the triple white. The aim of the work is to obtain marketable varieties exploiting the advantage agronomic given by heterosis.

## 2.3 Materials and methods

### 2.3.1 Experimental field

The experimental field is located in Tolentino (MC) at the CERMIS, research and experimentation centre for plant breeding (Coordinates 43 ° 23'69 " N, 13 ° 39'76 " E).

### 2.3.2. Plant materials

Plant materials used for the phenotypic characterization totalled 83 *V. faba* accessions, presented in Table 2.1.

The accessions are divided in two groups: 51 “triple white” accessions (white hilum, white seed coat, white flower) derived from a breeding program ISEA-ENEA for the improvement of Mediterranean faba bean (Bozzini and Chiaretti, 1997, 1999) and other 32 lines deriving from different selections.

#### *2.3.2.1 Origin of plant materials*

The 51 “triple-white” lines originate from a ENEA (Italian National Agency for New Technologies, Energy and Sustainable Economic Development) crossing program, with the aim the improvement of Mediterranean broad beans (Bozzini and Chiaretti, 1997).

**Tab. 2.1- Plant Material. The lines are shown with codes and not with Pedigree method names, for confidentiality agreements to protect the intellectual property of the owners of the breeding material, the company ISEA - Agroservice and ENEA.**

| # | CODE | Origin of the line | Botanical Variety | Generation | Flower coloration | Seed Colour | Hilum coloration |
|---|---|---|---|---|---|---|---|
| ***Group 1: Breeding program ISEA-ENEA*** | | | | | | | |
| 1 | **IS28** | Enea | *V. faba major* | F11 | Absent | Beige | Absent |
| 2 | **IS29** | Enea | *V. faba major* | F11 | Absent | Beige | Absent |
| 3 | **IS30** | Enea | *V. faba major* | F11 | Absent | Beige | Absent |
| 4 | **IS31** | Enea | *V. faba major* | F11 | Absent | Beige | Absent |
| 5 | **IS32** | Enea | *V. faba major* | F11 | Absent | Grey-beige | Present |
| 6 | **IS33** | Enea | *V. faba major* | F11 | Absent | Beige | Absent |
| 7 | **IS34** | Enea | *V. faba major* | F11 | Absent | Grey-beige | Absent |
| 8 | **IS35** | Enea | *V. faba major* | F11 | Absent | Beige | Absent |
| 9 | **IS36** | Enea | *V. faba major* | F11 | Absent | Green | Absent |
| 10 | **IS37** | Enea | *V. faba major* | F11 | Absent | Green | Absent |
| 11 | **IS38** | Enea | *V. faba major* | F11 | Absent | Green | Absent |
| 12 | **IS40** | Enea | *V. faba major* | F11 | Absent | Green | Absent |
| 13 | **IS41** | Enea | *V. faba major* | F11 | Absent | Grey-beige | Absent |
| 14 | **IS42** | Enea | *V. faba major* | F11 | Absent | Grey-beige | Absent |
| 15 | **IS46** | Enea | *V. faba major* | F11 | Absent | Beige | Absent |
| 16 | **IS47** | Enea | *V. faba major* | F11 | Absent | Beige | Absent |
| 17 | **IS65** | Enea | *V. faba major* | F7 | Absent | Green | Absent |
| 18 | **IS66** | Enea | *V. faba major* | F7 | Absent | Green | Absent |

| # | CODE | Origin of the line | Botanical Variety | Generation | Flower coloration | Seed Colour | Hilum coloration |
|---|---|---|---|---|---|---|---|
| 19 | **IS67** | Enea | *V. faba major* | F7 | Absent | Beige | Absent |
| 20 | **IS68** | Enea | *V. faba major* | F7 | Absent | Beige | Absent |
| 21 | **IS69** | Enea | *V. faba major* | F7 | Absent | Green | Absent |
| 22 | **IS70** | Enea | *V. faba major* | F7 | Absent | Green | Absent |
| 23 | **IS71** | Enea | *V. faba major* | F7 | Absent | Beige | Absent |
| 24 | **IS72** | Enea | *V. faba major* | F7 | Absent | Green | Absent |
| 25 | **IS73** | Enea | *V. faba major* | F7 | Absent | Green | Absent |
| 26 | **IS74** | Enea | *V. faba major* | F7 | Absent | Beige | Absent |
| 27 | **IS76** | Enea | *V. faba major* | F7 | Absent | Beige | Absent |
| 28 | **IS77** | Enea | *V. faba major* | F7 | Present | Beige | Absent |
| 29 | **IS60** | Enea | *V. faba major* | F7 | Absent | Green | Absent |
| 30 | **IS61** | Enea | *V. faba major* | F7 | Absent | Green | Absent |
| 31 | **IS62** | Enea | *V. faba major* | F7 | Absent | Green | Absent |
| 32 | **IS63** | Enea | *V. faba major* | F7 | Absent | Grey-beige | Absent |
| 33 | **IS64** | Enea | *V. faba major* | F7 | Absent | Beige | Absent |
| 34 | **IS78** | Enea | *V. faba major* | F7 | Absent | Beige | Absent |
| 35 | **IS79** | Enea | *V. faba minor* | F10 | Absent | Grey-beige | Absent |
| 36 | **IS80** | Enea | *V. faba minor* | F10 | Absent | Grey-beige | Absent |
| 37 | **IS81** | Enea | *V. faba minor* | F10 | Absent | Beige | Absent |
| 38 | **IS82** | Enea | *V. faba minor* | F10 | Absent | Beige | Absent |
| 39 | **IS83** | Enea | *V. faba minor* | F10 | Absent | Green | Absent |
| 40 | **IS48** | Enea | *V. faba major* | F7 | Absent | Beige | Absent |

| # | CODE | Origin of the line | Botanical Variety | Generation | Flower coloration | Seed Colour | Hilum coloration |
|---|---|---|---|---|---|---|---|
| 41 | **IS49** | Enea | *V. faba major* | F7 | Absent | Grey-beige | Absent |
| 42 | **IS50** | Enea | *V. faba major* | F7 | Absent | Grey-beige | Absent |
| 43 | **IS51** | Enea | *V. faba major* | F7 | Absent | Beige | Absent |
| 44 | **IS52** | Enea | *V. faba major* | F7 | Absent | Beige | Absent |
| 45 | **IS53** | Enea | *V. faba major* | F7 | Absent | Beige | Absent |
| 46 | **IS54** | Enea | *V. faba major* | F7 | Absent | Beige | Absent |
| 47 | **IS55** | Enea | *V. faba major* | F7 | Absent | Beige | Absent |
| 48 | **IS56** | Enea | *V. faba major* | F7 | Absent | Grey-beige | Absent |
| 49 | **IS57** | Enea | *V. faba major* | F7 | Absent | Beige | Absent |
| 50 | **IS58** | Enea | *V. faba major* | F7 | Absent | Beige | Absent |
| 51 | **IS59** | Enea | *V. faba major* | F7 | Absent | Beige | Absent |
| ***Group 2: Different selections*** | | | | | | | |
| 52 | **IS16** | Enea/Isea | *V. faba equina* | | Present | Beige | Present |
| 53 | **IS11** | Enea/Isea | *V. faba equina* | | Present | Beige | Present |
| 54 | **IS15** | Enea/Isea | *V. faba equina* | | Present | Beige | Present |
| 55 | **IS2** | Enea/Isea | *V. faba minor* | | Present | Black | Present |
| 56 | **IS10** | Enea/Isea | *V. faba minor* | | Present | Black | Present |
| 57 | **IS13** | Enea/Isea | *V. faba major* | | Present | Green | Present |
| 58 | **IS4** | Enea/Isea | *V. faba minor* | | Present | Green | Present |
| 59 | **IS5** | Enea/Isea | *V. faba equina* | | Present | Beige | Absent |
| 60 | **IS8** | Enea/Isea | *V. faba equina* | | Present | Beige | Present |
| 61 | **IS3** | Enea/Isea | *V. faba major* | | Present | Beige | Present |

| # | CODE | Origin of the line | Botanical Variety | Generation | Flower coloration | Seed Colour | Hilum coloration |
|---|---|---|---|---|---|---|---|
| 62 | **IS21** | Enea/Isea | *V. faba minor* | | Present | Beige | Present |
| 63 | **IS19** | Enea/Isea | *V. faba major* | | Present | Beige | Absent |
| 64 | **IS20** | Enea/Isea | *V. faba major* | | Present | Beige | Absent |
| 65 | **IS14** | Enea/Isea | *V. faba equina* | | Present | Grey-beige | Present |
| 66 | **IS6** | Enea/Isea | *V. faba minor* | | Present | Black | Present |
| 67 | **IS7** | Enea/Isea | *V. faba major* | | Present | Beige | Present |
| 68 | **IS18** | Enea/Isea | *V. faba equina* | | Present | Beige | Present |
| 69 | **IS43** | Enea | *V. faba equina* | | Absent | Grey-beige | Absent |
| 70 | **IS17** | Enea/Isea | *V. faba equina* | | Present | Beige | Present |
| 71 | **IS75** | Enea | *V. faba minor* | | Present | Beige | Present |
| 72 | **IS9** | Enea/Isea | *V. faba equina* | | Present | Green | Present |
| 73 | **IS12** | Enea/Isea | *V. faba minor* | | Present | Beige | Present |
| 74 | **IS1** | Enea/Isea | *V. faba equina* | | Present | Beige | Absent |
| 75 | **IS44** | Enea | *V. faba equina* | | Absent | Green | Absent |
| 76 | **IS45** | Enea | *V. faba minor* | | Absent | Green | Absent |
| 77 | **IS39** | Enea | *V. faba equina* | | Absent | Grey-beige | Absent |
| 78 | **IS26** | Enea | *V. faba minor* | | Present | Black | Present |
| 79 | **IS27** | Enea | *V. faba minor* | | Present | Black | Present |
| 80 | **IS22** | Enea | *V. faba minor* | | Present | Black | Present |
| 81 | **IS23** | Enea | *V. faba minor* | | Present | Black | Present |
| 82 | **IS24** | Enea | *V. faba minor* | | Present | Black | Present |
| 83 | **IS25** | Enea | *V. faba minor* | | Present | Black | Present |

Since 1991, more than 100 among cultivars, varieties and populations of *Vicia faba* L., from Italy, Europe, North Africa, Sudan, Ethiopia, Middle East, North and South America, China and Japan, have been collected, characterized and bred in protection (in greenhouses or screen houses in insect proof) at the experimental centre of ENEA at Casaccia (RM, Italy).

The material was kept in strict isolation during the flowering period, to avoid the interference of bees and bumble bees. This way, the whole seed is obtained from self-pollinated flowers (Fig. 2.1).

**Fig. 2.1 Faba bean lines under insect proof tunnel**

The lines have been selected over the years, according to the basic objectives: to combine the most advanced inbred lines of Northern Europe with the best lines from Italy and the Mediterranean basin that are more adapted to this microclimate. Hence, the goal was to introdusing interesting traits, such as a high level of self-fertility and precocity, which are present in the almost primitive lines grown in semi-arid areas of North Africa, the Middle East and Ethiopia (Bozzini and Chiaretti, 1997). Other traits of interest are white flower, white hilum and white seed coat (the so-called "triple white").

Initially 46 crossings were performed, choosing as "base" lines those from the Mediterranean:

- for *V. faba ssp. minor*, the Italian cultivars Manfredini and Vesuvio;

- for *V. faba ssp. equina*, the field bean, the cultivars Sikelia and Sicania, the dark green seed selected lines VHE 91, V35/84, V32/84, and the northern European cultivars Metissa and Statissa;

- for *V. faba ssp. major*, the Spanish cultivar Otono, the Sicilian Leonforte, Supersimonia and a selection from the ENEA Research Institute Trisaia (Rotondella, MT).

And as donors of the white flower trait, the cultivars Albatross, Caspar, Cresta, Grenny, Nerone, Optica, Sel. 5, Star, Toret, Trevoltebianca and Veritas were chosen.

At the same time, a specific breeding program was conducted for the development of highly self-fertile lines, with the closed flower (CF) trait. The chosen parental were: the Ethiopian open-flower line (OP) CGN 7751 (modest height, early development, high and stable level of self-fertility); four high production European OP lines, Veritas, Frinebo, Trigabo and Vesuvio; as female partners, lines from a PBI (Cambridge) selection, with the closed flower trait (CF) however associated with a very low self-fertility level.

The results showed that in the F2 generation, the traits “closed flower” (CF) and high level of self-fertility, have recombined, and the recombinants retain both traits in subsequent generations (Bozzini and Chiaretti, 1999).

Since 2010, the ENEA materials, were managed by the seed company ISEA Srl, which carries a selection and improvement program with the aim to constitute marketable lines with the traits of the absence of tannins ("triple white"), mainly self-fertile, and with the closed flower; at the time the material is made from 51 to white flower lines, from generations F7 to F11.

The remaining 32 lines of faba bean, (*Vicia faba minor, equina and major*), from generation F3 to F6, used for this characterization, originate from selections and crossings not known. In this group, we can find triple white lines and coloured lines, of widely varying dimensions and yield, phenotypically not stable.

### 2.3.3. Phenotyping

The accessions were sown in rows, 1 meter long, from the seed of a single plant, in the field under insect-proof tunnel. The distance was 30 cm between the rows and 15 cm between the plants within the row.

For 3 years, 23 phenotypic traits were detected in the field on the plants, and in the laboratory on dry seeds, following the UPOV (International Union for the Protection of New Varieties of Plants) provisions (2002) for the characterization of plants of *Vicia faba* L., with reference to the phenological stages described by Meier (Meier, 1997).

The UPOV, is an intergovernmental organization based in Geneva, Switzerland, created by the International Convention for the protection of new varieties (International Convention for the Protection of New Varieties of Plants). The Convention was adopted in Paris in 1961 and revised in 1972, 1978 and 1991.

In addition to these traits established by the UPOV protocol, were also detected: flower closure (for closed flower lines); number of pods per plant; villosity of the pod; number of seeds per pod. The traits are listed in table 2.3.

**Tab.2. 3. The list of the traits collected.**

| Trait | Initials | UPOV | Cluster |
|---|---|---|---|
| Foliage: intensity of green colour | **FC** | Main | Colouring |
| Seed: colour of seed coat | **SC** | Main | Colouring |
| Seed: pigmentation of the hilum | **SH** | Main | Colouring |
| Stem: anthocyanin coloration | **SAC** | Main | Colouring |
| Standard: anthocyanin coloration | **StAC** | Main | Colouring |
| Standard: extent of anthocyanin coloration | **StEAC** | Main | Colouring |
| Wing: colour of melanin spot | **WMC** | Main | Colouring |
| Wing: melanin spot | **WM** | Main | Colouring |
| Time of flowering (50% of the plants with at least one flower opened) | **FT** | Main | Earliness |
| Dry seed: shape of median longitudinal section | **SS** | Main | Morphology |
| Flower: closure | **CF** | No | Morphology |
| Flower: length | **FL** | Main | Morphology |
| Leaflet: length (basal pairs of leaflets at secondary node) | **LL** | Main | Morphology |
| Leaflet: position of maximum width (basal pairs of leaflets at secondary node) | **LMW** | Main | Morphology |
| Leaflet: width (basal pairs of leaflets at secondary node) | **LW** | Main | Morphology |
| Plant: growth type | **GT** | Main | Morphology |
| Plant: height | **H** | Main | Morphology |
| Pod: length | **PL** | Main | Morphology |
| Pod: villosity | **PV** | Secondary | Morphology |
| Pod: width | **PW** | Main | Morphology |
| 100 seeds weight | **100SW** | No | Yield |
| Number of pod per plant | **NPP** | No | Yield |
| Number of seeds per pod | **NSP** | No | Yield |

### *2.3.3.1. Phenotyping: description of traits*

UPOV, in an official publication of 2002 (updated in 2016), establishes a series of reliefs to be carried out with the crop in the field, with reference to the phenological phases of the plant described by Meier in 1997, to identify uniformity and differences between *Vicia faba* L. lines.

I) Intensity of green Colour of the Foliage (FC): it can be seen from the opening of the sixth leaf until the hatching of the first flower. Is used a scale of values from 1 to 3:

1 Light green (ie. Tista, Hiverna, Buzz);

2 Medium green (ie. Gloria, Wizard, Babylon);

3 Dark green (i.e. Maris Bead, Sultan);

II) Time of Flowering (TF): from 1 April (or March), are carried out field surveys every 2 days and recorded the estimated date of flowering, when the 50% of the plants has at least one flower opened. Established the date, in the analysis, it will be converted to a numeric value that represents the number of days from 1 April. For example, a line which blooms April 20 has the value 19.

III) Anthocyanin Coloration of the Stem(SAC): is detected by the appearance of the first flower until the one of the pods. In varieties with

presence of tannins (Fig. 2.2), you can observe one reddish coloration of the stem, which is evaluated with a scale:

3 Weak (i.e. Pistache, Divine, Trumpet, Arthur);

5 Medium (i.e. Victor, Scoop, Wizard);

7 Strong (i.e. Griffin).

**Fig.2.2 Anthocyanin coloration of the plant. To the left a coloured plant, to the right a white plant.**

IV) Leaflet Length (LL): is detected from the appearance of the first flower to full bloom. It should be observed the pair of basal leaves at the second node flowered; if there is difference in size between the pairs

of the leaves, to be considered the one that has greater dimensions. The leaf can be:

3 Short (i.e. Pistache, Delta, Maris Bead);

5 Medium (i.e. Victor, Tempest, Buzz);

7 Long (i.e. Limbo, Vertigo, Honey).

V) Leaflet Width (LW): see above. The leaf can be:

3 Narrow (i.e. Castel, Maris Bead, Bumble);

5 Medium (i.e. Columbo, Karl, Fury, Thor);

7 Broad (i.e. Condor, Honey).

VI) Position of Maximum Width of the lower Leaves (LMW.): see above. The maximum width can be, as shown in Figure 2.3:

1 Towards the tip of the leaf (i.e. Pistache, Boxer);

2 At the middle (i.e. Signal, Lynx, Wizard);

3 Towards the base (i.e. Victor, Griffin).

VII) Flower Length (FL): the data must be noted in full bloom, with all the other data on flower. The length is measured by stretching the flower as in Figure 2.4. This can be:

3 Short (i.e. Pistache, Maris Bead, Griffin);

5 Medium (i.e. Caspar, Fuego, Tundra);

7 Long (i.e. Victor, Fury, Sultan).

VIII) Flower Closure (CF): if present, is evaluated with reference to a scale from 1 to 5, where 1 is a little closed and 5 is perfectly closed.

IX) Melanin spots on flower Wings (WM): the spots may be present (i.e. Victor, Trumpet) or absent (i.e. Caspar). They can also be coloured (WMC):

1 Brown (i.e. Goldrush);

2 Black (i.e. Condor, Trumpet);

3 Greenish- yellow.

X) Anthocyanin Coloration of the Stendard (StAC): this observation must be done on the inner side of the banner. It may be present (i.e. Pistache, Condor) or absent (i.e. Caspar); if present, can be more or less extended (StEAC) (refer to Figure 2.5):

1 Small (Pistache, Fuego, Honey);

2 Medium (i.e. Hiverna, Scoop, Sultan);

3 Large (i.e. Arthur).

XI) Growth Type (GT) and Height (H) of the plant: this observation can be done after the stage of withering, at any stage of pod development,

but before it reaches its final length. The plant can have a growth type determined (i.e. Tista) or indeterminate (i.e. Condor) (see figure 2.6). Regarding the height, the plant may be:

1 Low (i.e. Pistache, Babylon, Sultan);

2 Medium (i.e. Columbo, Fuego, Buzz);

3 High (i.e. Condor, Lynx, Bumble).

The second phase of the reliefs was made in the laboratory, after the plant has dried and the pods were harvested.

For each accession, the whole pods from a sample of six plants have been collected, randomly selected within the row, avoiding the header plants (the first and the last) that would lead to a distorted result, due the board effect.

Data collected in the post-harvest are:

XII) Number of Pods per Plant (NPP): the average of the counts of the pods of the six plants of the sample.

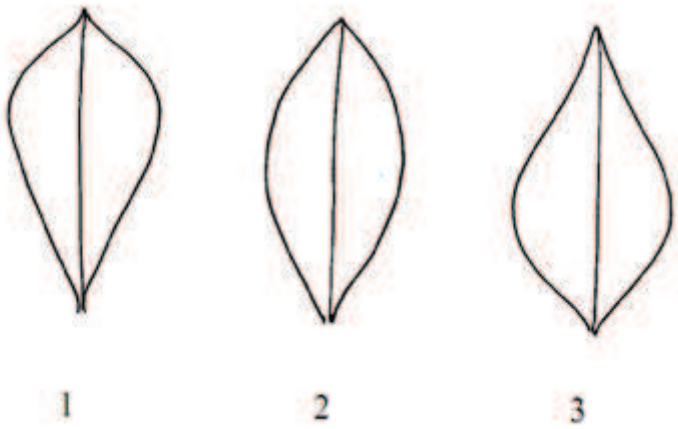


**Fig.2.3. Position of the maximum width of the basal leaf (LMW).**

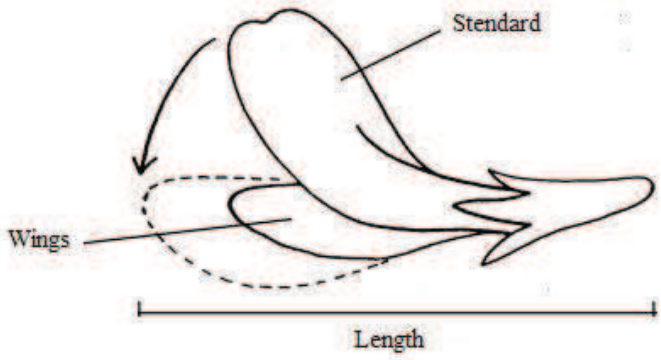


**Fig. 2.4 Determination of the length of the flower (FL) of *V. faba* L.**

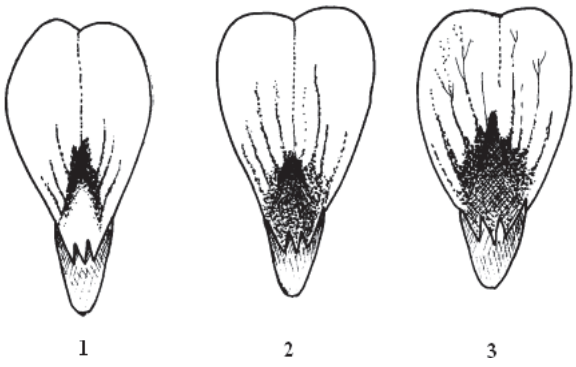


**Fig. 2.5 Stendard: extent of anthocyanin coloration (StEAC).**


**Fig. 2.6 Growth type (GT) of *Vicia faba* plants.**

XIII) Pod Length (PL): should be determined without considering the final curved beak. The pod can be:

1 Very short (i.e. Maris Bead);

2 Short (i.e. Condor, Fury);

3 Medium (i.e. Gloria, Boxer, Griffin);

4 Long (i.e. Caspar, Vasco, Babylon, Wizard).

XIV) Pod Width (PW): it is estimated observing the width, suture to suture. The pod can be:

1 Narrow (i.e. Condor, Lynx);

2 Medium (i.e. Pistache, Scoop, Sultan);

3 Broad (i.e. Victor, Bumble).

XV) Villosity of the Pod (PV): is considered by UPOV as a secondary character for phenotyping. In this study, the pod has been evaluated as:

1 Smooth;

2 Slightly hairy;

3 Hairy.

XVI) Number of seeds per plant (NSP): the average of the counts of the seeds of the six plants of the sample.

XVII) Shape of median longitudinal section of the dry seed (SS): to evaluate this trait, observe Figure 2.7 as reference. The seed can be of the form:

1 Circular (i.e. Maris Bead);

2 Elliptical (i.e. Condor);

3 Irregular (i.e. Columbo, Fury, Bumble).

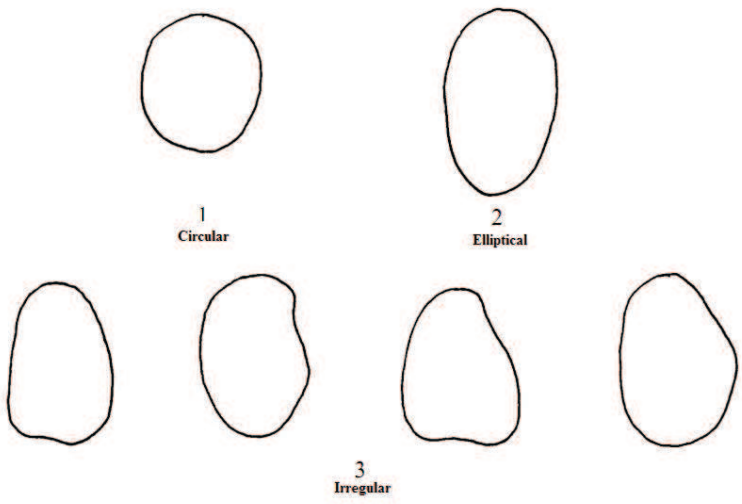


**Fig. 2.7 Shape of median longitudinal section of the seeds (SS) of *Vicia faba* L.**

XVIII) Weight of 100 seeds (100SW): expressed in grams. The seeds are counted, 100 or multiples, and the weight is noted.

XIX) Seed: colour of the seed coat (SC): the evaluation should be carried out immediately after harvest, because seeds with clear integument, in case of tannin content may darken, when too much time passes from the harvest. The seed can be:

1 Beige (i.e. Condor, Trumpet, Wizard);

2 Grey-beige (i.e. Caspar);

3 Green (i.e. Palacio);

4 Black (i.e. Tyrol).

XX) Pigmentation of the hilum (SH): trait correlated with the presence of tannins, vicine and convicine. SH can be stated as present (i.e. Condor, Maris Bead, Clipper) or absent (i.e. Victor, Trumpet, Wizard). A tolerance range of 5% is permitted.

### *2.3.3.2 Analysis of genetic similarity and principal component analysis (P.C.A.) based on phenotypic traits.*

The data on which was conducted the statistical analysis are the harmonics averages of the phenotypic reliefs of years 2010-2011, 2011-2012 and 2012-2013.

We chose to use harmonic average for the analysis, in order to reduce any bias and because for some lines was not possible to detect all the data for the year 2012-2013.

The data of the traits object of evaluation, have been subjected to frequency analysis; for each one, the mean and the standard deviation was calculated.

All statistical analyses were conducted through the JMP 8 software (SAS Campus Drive, Cary, USA).

In the matrices used to calculate the genetic similarity (GSij) between the possible pairs of accessions analysed, were considered all the phenotypic traits detected. The reliefs of non-numeric traits were converted, for the analysis, on a numerical scale that goes from 1 to N, with N equal to the number of possible variants.

The similarity between all possible pairs of accessions were calculated using the similarity coefficient "simple matching", for the traits that have two or more alternatives: proportion of traits that have the same variant.

This coefficient was calculated with the following formula:

$$GS_{ij} = \frac{m}{n}$$

where *m* = number of the character variants that show correlation of their state, that is, the same variant in i and j (in our case, is equal to the number of traits for which the two accessions compared have the same variant; for example, both have the combination "colour foliage: medium" and "shape of median longitudinal section: irregular") and *n* = total number of comparable traits.

We also did a Principal Components Analysis (P.C.A.). The concept that provides the basis of the principal components is to reduce the number of variables in the data set starting, in order to obtain the new variables, not related to each other, and with a wider variance.

That is done through the linear transformation of the variables, which projects the original ones in a new Cartesian system in which the variables are sorted in order of decreasing variance: therefore, the variable that has the greatest variance is projected on the first axis, the second on the second axis, and so on.

### 2.3.4 Agronomic evaluation

In the year 2011/2012, an experimental test was performed to evaluate the 83 lines at agronomic level.

The experimental design chosen for the test is the Augmented Block Design Scheme (Youden, 1951). This scheme is useful in those cases in which there is poor availability of a seeds to be able to perform a

replicated test. Furthermore, it is useful when in the field there could be problems of environmental heterogeneity (in particular of the soil), in one direction.

| | | | | | | |
|---|---|---|---|---|---|---|
| IS22 | IS23 | IS24 | IS25 | TEST1 | Filler | Block 3 |
| IS27 | IS26 | IS39 | IS45 | IS44 | IS1 | |
| IS18 | IS43 | IS17 | IS75 | IS9 | IS12 | |
| TEST2 | IS7 | IS6 | IS14 | IS20 | IS19 | |
| IS4 | IS5 | IS8 | IS13 | IS3 | IS21 | |
| IS10 | IS2 | IS15 | IS11 | IS16 | IS34 | Block 2 |
| IS54 | IS55 | IS56 | IS57 | IS58 | IS59 | |
| IS52 | IS52 | IS51 | IS50 | IS49 | IS48 | |
| IS79 | IS80 | IS81 | IS82 | IS83 | TEST2 | |
| IS78 | TEST1 | IS64 | IS62 | IS62 | IS61 | |
| IS72 | IS73 | IS74 | IS76 | IS77 | IS60 | Block 1 |
| IS71 | IS70 | IS69 | IS68 | IS67 | IS66 | |
| IS41 | IS42 | IS46 | IS47 | TEST2 | IS65 | |
| IS40 | IS39 | IS38 | IS37 | IS36 | TEST1 | |
| IS28 | IS29 | IS30 | IS31 | IS32 | IS33 | |

**Fig. 2.8 Experimental scheme (AB design) of the field test.**

The lines are arranged in plots of 10 m², according to the experimental scheme shown in Figure 2.8. For reasons of space, our test was performed according to a scheme 6 x 15, that is, the basic unit of 6 parcels repeated for 15 files. This has meant that there was a disproportion between length and width of the plot area, implying, therefore, heterogeneity occurs along the longer side.

The Augmented Block (AB) design is a Linked Block (LB) design, arranged to have *b* blocks, each of size *k*, which must have the following characteristics:

- Each treatment is at maximum in a block;
- Treatments are to be distributed in equal (*k*) number in all blocks;
- Each pair of blocks has exactly one $\mu$ number of varieties that are equal.

The AB design allows to evaluate the effect of the block, and consequently the effect of the genotype of the examined lines, without having the replicas, but by calculating the variability on the controls, which are repeated in each block and randomly assigned in the same block.

The experimental model is represented by this function:

$$y_{ij=}\mu + \beta_i + c_j + t_{k(i)} + \varepsilon_{ij}$$

where:

$c$ (=$v$) = number of controls;

$t$ (=$v$*) = number of lines;

$b$ = number of blocks;

$k$ = size of the block;

$\mu$ = number of identical lines for each block;

$l_i$= number of lines per block;

$\varepsilon$ = standard error.

The constants $v$, $k$, $r$, $b$ and $\mu$, are the LB design parameters, associated with the test varieties.

$y_{ij}$ is the production of the $j$th block, where $j$ = 1, 2, 3, ….. $b$, and $\beta_j$ is the total value of the $j$th block.

The v* lines to be evacuate, should be randomized so that $l_j$ (number of varieties in the $j$th block) lines are in the $j$th block, so $\sum_j = 1^r = v*$, where $r$ è is the number of replicas of the variety test.

$z_{ij}$ is the production of the $i$th line, where $i$ = 1, 2, 3, ….. $i$, in the $j$th block, where $j$ = 1, 2, 3, ….. $b$.

For simplicity of the model, it is assumed that the block is not subject to effects *ad random* (due to chance).

The statistical analysis is performed on a complete randomized block, and plans to estimate the Mean Squared Error (MSE) using the variety test (v) (MSE controls). The MSE is calculated as:

$$MSE = \frac{\sum_1^n (v - \bar{v})^2}{n}$$

Where:

$v$ = value of the controls;

$\bar{v}$ = the average of the control values;

$n$ = total number of controls.

The difference between the *i*th and the *i'*th line in the *j*th block is evaluated as $z_{ij} - z_{i'j}$ with a standard error given by: $\sqrt{2 * MSE}$.

The difference between the *i*th and the *i'*th line in *j* different blocks $(j \neq j')$ is valued as $z_{ij} - z_{i'j'} - (\bar{y}_j - \bar{y}_{j'})$ where $\bar{y}_j$ is the *j*th block average, calculated on the production of the test varieties $(v)$,with a standard error given by:

$$\sqrt{(2 * (v + 1) * \frac{MSE}{v})}$$ (Federer, 1975)

In our case:

c=2 (*Scuro di Torrelama* and *Chiaro di Torrelama*);

t=84 (83 lines + 1 filler);

b=3;

μ= 2;

$l_i$=28;

k=30.

The total number of plots, so, it is 90.

### 2.3.5 Synthetic varieties

From our population of inbred lines were chosen the best genotypes, selected for their agronomic and morphologic traits. In selecting the parental combinations, we evaluated:

- the availability of seed;
- membership to the clusters identified by the combination of the data of phenotypic and agronomic evaluation;
- the almost identical phenotype;
- historical data available through the pedigree method.

Plant materials used for the creation of the syntetic lines is a part of the population, including 26 triple white *V. faba* accessions, presented in Table 2.3. The lines are divided into 4 different groups, depending on their Pedigree, as indicated in the table.

The method that was used for obtaining the synthetic varieties is summarized in the diagram in figure 2.9, reworked by the scheme for obtaining synthetic varieties of Barcaccia and Falcinelli (2006).

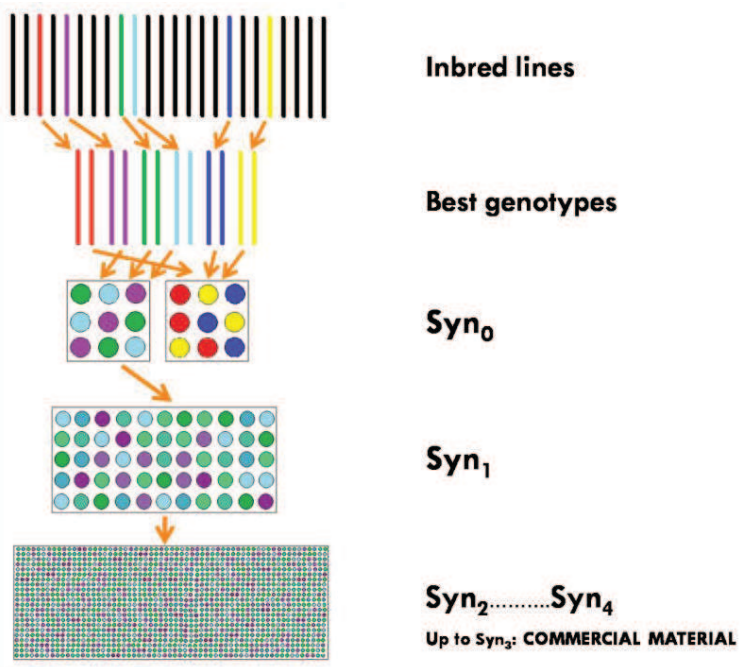


**Fig. 2.9. Diagram of the process to obtain a synthetic variety of Faba bean.**

**Tab2.3. Plant material.**

| Pedigree Group | Code | Generation | Botanical variety |
|---|---|---|---|
| G1 | **IS28** | F11 | *V. faba major* |
| G1 | **IS29** | F11 | *V. faba major* |
| G1 | **IS31** | F11 | *V. faba major* |
| G1 | **IS42** | F11 | *V. faba major* |
| G1 | **IS46** | F11 | *V. faba major* |
| G2 | **IS67** | F7 | *V. faba major* |
| G2 | **IS63** | F7 | *V. faba major* |
| G2 | **IS64** | F7 | *V. faba major* |
| G3 | **IS78** | F10 | *V. faba minor* |
| G3 | **IS79** | F10 | *V. faba minor* |
| G3 | **IS80** | F10 | *V. faba minor* |
| G3 | **IS81** | F10 | *V. faba minor* |
| G3 | **IS82** | F10 | *V. faba minor* |
| G3 | **IS83** | F10 | *V. faba minor* |
| G4 | **IS48** | F7 | *V. faba major* |
| G4 | **IS49** | F7 | *V. faba major* |
| G4 | **IS50** | F7 | *V. faba major* |
| G4 | **IS51** | F7 | *V. faba major* |
| G4 | **IS52** | F7 | *V. faba major* |
| G4 | **IS53** | F7 | *V. faba major* |
| G4 | **IS54** | F7 | *V. faba major* |
| G4 | **IS55** | F7 | *V. faba major* |
| G4 | **IS56** | F7 | *V. faba major* |
| G4 | **IS57** | F7 | *V. faba major* |
| G4 | **IS58** | F7 | *V. faba major* |
| G4 | **IS59** | F7 | *V. faba major* |

These genotypes were multiplied in the field, under insect proof tunnel. The seed collected were mixed in different combinations (generation

Syn0). The mixtures were seeded and left to free pollination in the field, isolated from other crops.

The following year we collected the Syn1, which is the first real generation of synthetic. The following year Syn1 is, again, left to free pollination, and so on. At the generation Syn3, we already have marketable material.

The seeds collected each year, are divided into two parts, which are used respectively to:

- multiply the seed in isolation;
- perform a preliminary test to determine the agronomic value of the synthetic.

#### *2.3.5.1 Assessment of performances of synthetic varieties.*

For the preliminary assessment of the agronomic performance of synthetic varieties, two field trials were conducted in the year 2013-2014 (scheme in figure 2.10) and in 2014-2015 (scheme in figure 2.11), on plots of 7,5 $m^2$, per the Augmented Block Design scheme (Youden, 1951) (see Chapter 2.3.4). As a term of comparison, we used five commercial varieties (Chiaro di Torrelama, Scuro di Torrelama, Rumbo, Collameno, Protobat), and seven inbred lines (IS9, IS15, IS18, IS25, IS96, IS107, IS138), coloured and not.

**Fig. 2.10 Experimental design of agronomic field test of 2013-2014. The inbred lines are marked in blue; in red, the synthetic lines under test.**

| | | | |
|---|---|---|---|
| Collameno | Chiaro Torrelama | IS15 | Block 2 |
| IS-Sint3 | Rumbo | Scuro torrelama | |
| Chiaro Torrelama | IS25 | Collameno | |
| IS18 | Rumbo | Scuro torrelama | |
| IS-Sint1 | IS9 | Chiaro Torrelama | Block 1 |
| Chiaro Torrelama | IS107 | Rumbo | |
| Scuro torrelama | Collameno | IS-Sint2 | |
| Collameno | Rumbo | Scuro torrelama | |

**Fig. 2.11. Experimental design of agronomic field test of 2014-2015. The inbred lines are marked in blue; in red, the synthetic lines under test.**

| | | | |
|---|---|---|---|
| Protobat | IS-Sint5 | Chiaro Torrelama | Block 2 |
| IS138 | Scuro torrelama | Protobat | |
| Chiaro Torrelama | IS96 | Rumbo | |
| IS-Sint7 | Rumbo | Scuro torrelama | |
| Protobat | IS-Sint6 | Protobat | Block 1 |
| Rumbo | Chiaro Torrelama | Rumbo | |
| IS9 | Scuro torrelama | IS-Sint4 | |
| Chiaro Torrelama | IS18 | Scuro torrelama | |

## 2.4 Results

In this section are shown the phenotypic data collected in three years, the results of the statistical analysis made on it, the outcome of the agronomic evaluation of the 83 lines of the population and the results of the preliminary evaluation of 7 synthetic lines.

### 2.4.1 Phenotyping

#### *2.4.1.1 Frequency analysis and correlations of phenotypic traits*

We have two types of phenotypic traits: continuous and ordinal. Continuous traits are those whose data is detectable as a continuous numerical value, such as the flowering time or the 100 seeds weight. Ordinal traits are those who, for purposes of statistical analysis, has been assigned a numerical value that identifies a class of phenotypic variables.

Each one of the 83 lines was found to have a determined growth type.

Regarding the colour of the leaves, (FC), we observe a high prevalence (86.7%) of the “medium green” colour. Clearer leaves have been identified in 11 “triple-white” faba bean lines, and darker leaves in 7 coloured lines.

The average date of flowering (FT) is around 13 days from the first of April. The 60.3% of the lines FT is distributed fairly evenly between the 9 and the 17 days after, so they are all pretty precocious. Only 6 are

tardive lines (IS22, IS23, IS24, IS26, and IS25, all coloured lines with the same origin), FT are distributed from 20 to 22 days after April 1.

The results of the frequency analysis, of traits related to anthocyanin coloration of the plant, that is, the colouring of the stem (SAC), the presence of stains on flower wings (WM), their colour (WMC), the presence of anthocyanin coloration on the standard (StAC), the extending of the spot (StEAC), and black pigmentation of hilum (ST), show that 65% of the test lines appears to be in total absence of tannins, as the stem does not have reddish hues, the flower is completely white, as well as the hilum.

Almost all of the coloured lines show a weak or medium coloration of the stem; the melanin spots on the wings are brown for 65% of the cases and black for the remaining part; more than half of the coloured lines show a small or medium extension of the anthocyanin spot on the standard, about 6.7% of the population has a big extension.

About 80% of the lines has short length leaves or medium length leaves (LL), and 74% have large or medium wide leaves (LW), always with a slight prevalence of mid-size. The position of the maximum width of the leaf (LMW) is for almost all the lines at the centre, the 12% of the lines has the widest part of the leaf towards the tip and a single line is wider towards the base, IS20.

For the length of the flower (FL) prevails the average size; 10% of the lines has the long flower phenotype and 5% the short flower.

The closed flower trait (FC) was found only in three white flower lines: two lines at generation F11, with tightly closed flower (IS30, IS33) and one F7 line with less closed flower (IS49). The rest of the lines, 96%, is open-flower.

The height of the plant (H) is normally distributed, with a prevalence of lines with medium height, medium to high or medium to low. The accessions analysed tend to be high (63.86%). The 11% of the lines are high, and only 6 lines are short (IS10, IS28, IS46, IS60, IS77, IS80).

Also for the number of pods per plant (NPP), the values are distributed almost all around the mean, which is about 7 pods. Four lines, (IS42, IS60, IS76 and IS77), have an average NPP of about 1 pods per plant (the lowest detected value); only 2 lines have a high NPP, between 12 and 13: IS2 and IS21. The number of seeds per plant (NSP) values are all around the average of 12, with peaks of 7 (14%), 14 (12%) and in 21 (10%). The lowest value, reported for 3 lines, is about one seed per plant (IS60, IS62, IS76). A line, IS62, has a high NSP compared to the other lines: 33 seeds.

Regarding the length of the pod (PL), there is prevalence of lines with medium length pods. The lowest value measured, detected in four lines (IS60, IS61, IS62 and IS70), is about 4 cm. The 3% of lines appears to have pods with a length between 12 and 14 cm (three white lines, IS45, IS82, IS81).

The majority of accessions (56%) has medium and medium-large pods (PW); 32% of the lines has fairly tight pods, and 12% has tight pods.

The villosity of the pod (PV), a secondary trait for the characterization of *Vicia faba* L. accessions, is not of clear determination; most of the accessions have segregating plants with pods of different villosity. The 76% of the lines have slightly hairy pods, predominantly on the lines with hairless pod.

Almost all lines have the section of dry seed (SS) irregularly shaped, except 3 lines that have tended roundish seeds, (IS22, IS49, IS79) and 5 lines with elliptical seeds (IS6, IS16, IS16, IS45, IS78).

Analysing the frequencies of the 100 seeds weight (100SW) of accessions, is possible to observe that 72% of the lines has 100SW below 70 g, and therefore can be included in the commercial category of field beans (*V. faba minor*); the 21% to the category of horse beans (*V. faba equina*) has 100SW between 70 and 100 g; Three lines, IS9, IS20, IS48 has 100SW greater than 100 g, and can be counted among the broad beans (*V. faba major*). The average weight of the lines is 55 g, and the majority (31%) weigh between 60 and 70 g.

The 90% of the lines under consideration for the trait of the colour of the seed coat of the dry seed (SC), has a very light colour, beige, grey-beige or green. The remaining 10% have black seed coat (IS2, IS10, IS22, IS23, IS24, IS25, IS26, IS27). 28% of the lines has black

pigmentation of the hilum (SH), in the other accessions, the pigmentation is absent.

To analyse the similarity between the 83 accessions, was performed, using the JMP software 8 (http://www.jmp.com/software/jmp8/), a principal component analysis (PCA) and a two-way Cluster Analysis.

Table 2.4 is a correlation matrix that shows the individual values of correlation between pairs of traits; the significant values at 95% and 99% are highlighted.

The high correlation between some of the traits appears evident in figure 2.12, where the correlations are shown according to the p-value.

The p-value of a statistical hypothesis, also called significance level, indicates the probability of obtaining a result equal to or more extreme than the observed, supposed true the null hypothesis. The null hypothesis in our case is that values of the traits are related. In the colour map, for each pair of analysed traits is indicated, with a colour, the probability that the traits are correlated. The traits that are probably related to each other are indicated with shades of red, up to the level of significance of 5%, indicated with grey. Traits that are probably not related are indicated with shades of blue.

| | FC | FT | SAC | LL | LW | LMW | FL | CF | WM | WMC | STAC | STEAC | H | NPP | NSP | PL | PW | PV | SS | 100SW | SC | SH |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **FC** | | | | | | | | | | | | | | | | | | | | | | |
| **FT** | 0,06 | | | | | | | | | | | | | | | | | | | | | |
| **SAC** | ***0,31*** | *0,28* | | | | | | | | | | | | | | | | | | | | |
| **LL** | -0,08 | 0,08 | -0,12 | | | | | | | | | | | | | | | | | | | |
| **LW** | 0,16 | 0,04 | 0,12 | -0,13 | | | | | | | | | | | | | | | | | | |
| **LMW** | -0,07 | -0,10 | 0,05 | -0,08 | 0,09 | | | | | | | | | | | | | | | | | |
| **FL** | 0,21 | ***-0,45*** | 0,13 | -0,05 | 0,18 | -0,03 | | | | | | | | | | | | | | | | |
| **CF** | -0,13 | -0,13 | -0,13 | 0,13 | -0,16 | 0,07 | -0,05 | | | | | | | | | | | | | | | |
| **WM** | ***0,31*** | 0,33 | ***0,96*** | -0,12 | 0,07 | -0,05 | ***0,10*** | -0,14 | | | | | | | | | | | | | | |
| **WMC** | ***0,31*** | 0,34 | ***0,95*** | -0,13 | 0,08 | -0,04 | ***0,10*** | -0,14 | ***0,99*** | | | | | | | | | | | | | |
| **STAC** | ***0,31*** | 0,33 | ***0,96*** | -0,12 | 0,07 | -0,05 | ***0,10*** | -0,14 | ***1,00*** | ***0,99*** | | | | | | | | | | | | |
| **STEAC** | ***0,35*** | ***0,37*** | ***0,93*** | -0,11 | 0,07 | 0,03 | 0,07 | -0,13 | ***0,95*** | ***0,96*** | ***0,95*** | | | | | | | | | | | |
| **H** | 0,09 | ***0,50*** | ***0,41*** | 0,01 | 0,04 | -0,08 | -0,12 | -0,18 | ***0,41*** | ***0,42*** | ***0,41*** | ***0,43*** | | | | | | | | | | |
| **NPP** | 0,10 | 0,04 | ***0,41*** | 0,09 | 0,13 | 0,08 | 0,09 | -0,01 | ***0,38*** | ***0,39*** | ***0,38*** | ***0,36*** | *0,22* | | | | | | | | | |
| **NSP** | 0,19 | 0,03 | ***0,55*** | -0,02 | 0,03 | 0,06 | 0,12 | -0,03 | ***0,55*** | ***0,56*** | ***0,55*** | ***0,53*** | *0,27* | ***0,87*** | | | | | | | | |
| **PL** | 0,15 | ***-0,50*** | -0,11 | -0,06 | -0,02 | 0,15 | ***0,31*** | -0,05 | -0,13 | -0,14 | -0,13 | -0,15 | -0,10 | *0,25* | ***0,32*** | | | | | | | |
| **PW** | -0,12 | ***-0,32*** | *-0,49* | 0,16 | 0,03 | 0,04 | ***0,28*** | 0,16 | *-0,54* | *-0,54* | *-0,54* | -0,53 | ***-0,27*** | *-0,36* | -0,44 | ***0,16*** | | | | | | |
| **PV** | 0,10 | 0,10 | -0,10 | 0,17 | 0,08 | -0,01 | 0,06 | 0,08 | -0,14 | -0,12 | -0,14 | -0,11 | 0,11 | *0,23* | 0,14 | -0,01 | 0,17 | | | | | |
| **SS** | -0,09 | 0,07 | -0,05 | 0,17 | 0,18 | 0,01 | 0,01 | -0,20 | -0,05 | -0,05 | -0,05 | -0,08 | -0,05 | -0,07 | -0,13 | 0,00 | 0,22 | 0,16 | | | | |
| **100SW** | 0,19 | ***-0,33*** | ***0,42*** | -0,14 | 0,03 | 0,08 | ***0,36*** | -0,08 | ***0,40*** | ***0,40*** | ***0,40*** | ***0,39*** | 0,13 | ***0,36*** | ***0,52*** | ***0,67*** | ***-0,08*** | -0,18 | -0,11 | | | |
| **SC** | 0,04 | ***0,60*** | ***0,30*** | 0,02 | 0,08 | -0,09 | ***-0,30*** | -0,13 | ***0,39*** | ***0,40*** | ***0,39*** | ***0,38*** | ***0,31*** | 0,15 | *0,24* | ***-0,30*** | ***-0,49*** | -0,03 | -0,15 | -0,17 | | |
| **SH** | *0,23* | ***0,38*** | ***0,82*** | -0,09 | 0,09 | -0,08 | 0,03 | -0,12 | ***0,86*** | ***0,85*** | ***0,86*** | ***0,79*** | ***0,36*** | ***0,36*** | ***0,49*** | -0,10 | ***-0,54*** | -0,19 | -0,07 | ***0,30*** | ***0,49*** | |

**Tab. 2.4 Matrix of correlations between the traits. Bold: significant correlations at 95%; *Italics and bold*: significant correlation at 99%.** *FC= Foliage, intensity of green colour; FT= Time of flowering (50% of the plants with at least one flower opened); SAC= Stem: anthocyanin coloration; LL= Leaflet: length (basal pairs of leaflets at secondary node); LW= Leaflet: width (basal pairs of leaflets at secondary node); LMW= Leaflet: position of maximum width (basal pairs of leaflets at secondary node); FL= Flower: length; CF= Flower: closure; WM= Wings: melanin spot; WMC= Wings: colour of melanin spot; STAC= Stendard: anthocyanin coloration; STEAC= Stendard: extent of anthocyanin coloration; H= Plant: height; NPP= Number of pod per plant; NSP= Number of seeds per pod; PL= Pod: length; PW= Pod: width; PV= Pod: villosity; SS= Dry seed: shape of median longitudinal section; 100SW= 100 seeds weight; SC= Seed: colour of seed coat; SH= Seed: pigmentation of the hilum.*

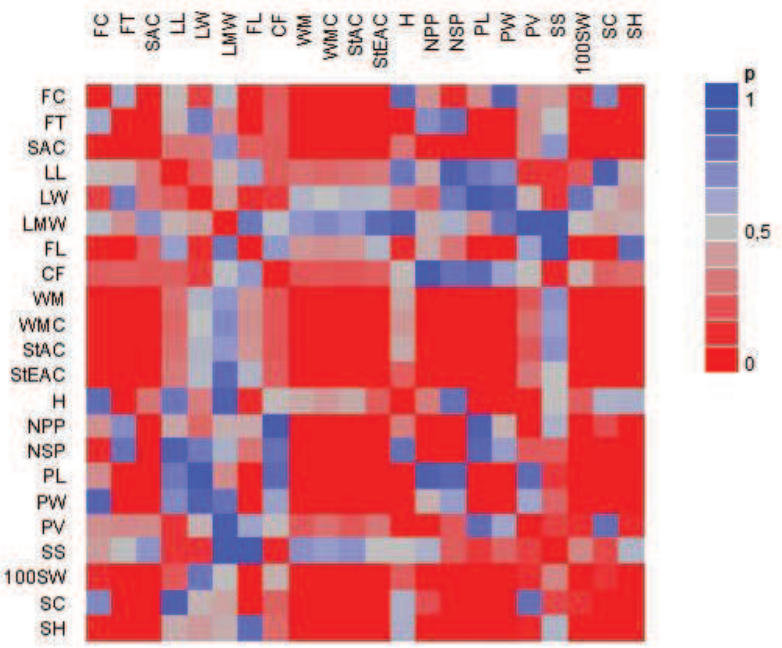


**Fig. 2.12. Coloured map on the correlation between the traits, based on p value**

*FC= Foliage, intensity of green colour; FT= Time of flowering (50% of the plants with at least one flower opened); SAC= Stem: anthocyanin coloration; LL= Leaflet: length (basal pairs of leaflets at secondary node); LW= Leaflet: width (basal pairs of leaflets at secondary node); LMW= Leaflet: position of maximum width (basal pairs of leaflets at secondary node); FL= Flower: length; CF= Flower: closure; WM= Wings: melanin spot; WMC= Wings: colour of melanin spot; STAC= Standard: anthocyanin coloration; STEAC= Standard: extent of anthocyanin coloration; H= Plant: height; NPP= Number of pod per plant; NSP= Number of seeds per pod; PL= Pod: length; PW= Pod: width; PV= Pod: villosity; SS= Dry seed: shape of median longitudinal section; 100SW= 100 seeds weight; SC= Seed: colour of seed coat; SH= Seed: pigmentation of the hilum.*

### *2.4.1.2 Principal Component Analysis (PCA)*

A Principal Component Analysis (PCA) has been carried out, using the JMP software: transforming the variables of 22 traits surveyed, which

projects the original ones in a new Cartesian system in which the new variable with the greatest variance is projected on the first axis, and the second variable, according to size of the variance, on the second axis.

The two main components express the greatest diversity of accessions and used as axes in a Cartesian plane, they show how the traits examined and the different accessions behave in relation to them. The graphics in figure 2.13 and 2.14, shows the choice of the main components according to the correlation between traits. The two components that have the highest Eigenvalue, (respectively 7.21 and 3.35) were chosen as Principal Component.

These will become the axis of the new Cartesian system as shown in Figures 2.15 and 2.17; they express 48% of the total variability of the lines under consideration. The figure 2.15 graphically shows the correlations found between the traits.

In the first quadrant, we observe a large number of vectors, representing the traits that appear to be more related to each other. Those that in the graphic are closest, are the traits linked to the presence of tannins in the plant (in some cases, the vectors overlap). Also, the number of seeds per pod, per plant and the weight of 100 seeds appears to be correlated. Apart from these, we find the length of the flower and the width of the basal leaf. In the second quadrant, the vectors of the flowering date, the height of the plant, the colour of the seed coat and the black pigmentation of hilum can be observed.

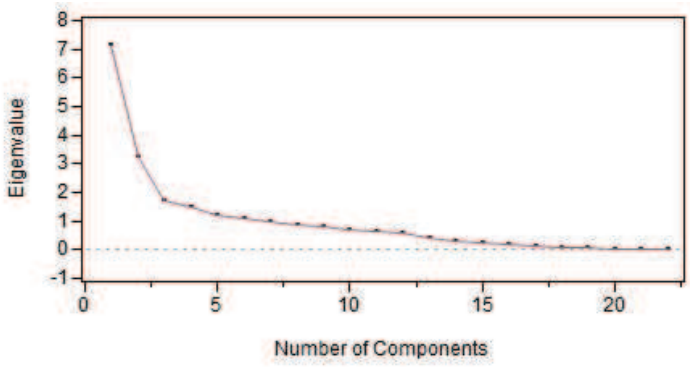


**Fig. 2.13: Scree plot: Eigenvalue analysis for PCA analysis. The first two components from the left are those that exert the greater variability.**

| Number | Eigenvalue | Percent | 20 40 60 80 | Cum Percent |
|---|---|---|---|---|
| 1 | 7,1682 | 32,583 | | 32,583 |
| 2 | 3,2549 | 14,795 | | 47,378 |
| 3 | 1,7184 | 7,811 | | 55,189 |
| 4 | 1,5160 | 6,891 | | 62,080 |
| 5 | 1,1949 | 5,431 | | 67,511 |
| 6 | 1,1001 | 5,000 | | 72,512 |
| 7 | 1,0046 | 4,566 | | 77,078 |
| 8 | 0,8881 | 4,037 | | 81,115 |
| 9 | 0,8207 | 3,730 | | 84,845 |
| 10 | 0,7001 | 3,182 | | 88,028 |
| 11 | 0,6448 | 2,931 | | 90,959 |
| 12 | 0,5822 | 2,646 | | 93,605 |
| 13 | 0,3948 | 1,795 | | 95,400 |
| 14 | 0,3179 | 1,445 | | 96,845 |
| 15 | 0,2238 | 1,017 | | 97,862 |
| 16 | 0,1763 | 0,802 | | 98,664 |
| 17 | 0,1135 | 0,516 | | 99,180 |
| 18 | 0,0853 | 0,388 | | 99,567 |
| 19 | 0,0511 | 0,232 | | 99,800 |
| 20 | 0,0404 | 0,184 | | 99,983 |
| 21 | 0,0037 | 0,017 | | 100,000 |
| 22 | 0,0000 | 0,000 | | 100,000 |

**Fig. 2.14 Results of PCA based on the Correlations: Principal Components**

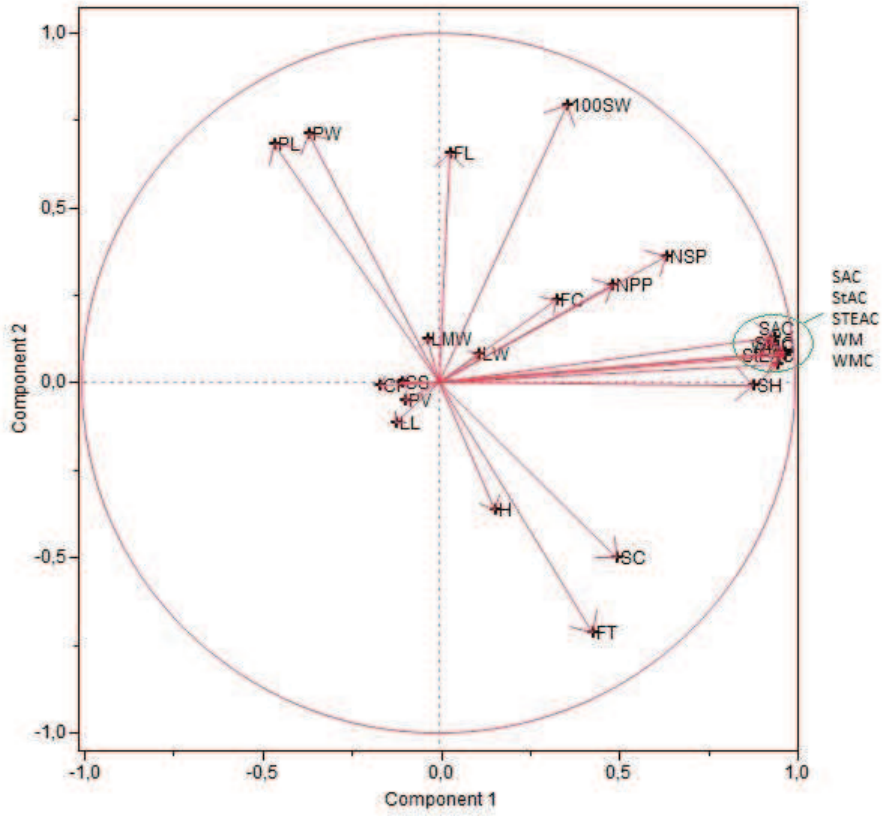


**Fig. 2.15 Result of the PCA Analysis on the 83 accessions. Disposal of traits in relation to the two Principal Components selected.** *FC= Foliage, intensity of green colour; FT= Time of flowering (50% of the plants with at least one flower opened); SAC= Stem: anthocyanin coloration; LL= Leaflet: length (basal pairs of leaflets at secondary node); LW= Leaflet: width (basal pairs of leaflets at secondary node); LMW= Leaflet: position of maximum width (basal pairs of leaflets at secondary node); FL= Flower: length; CF= Flower: closure; WM= Wings: melanin spot; WMC= Wings: colour of melanin spot; STAC= Standard: anthocyanin coloration; STEAC= Standard: extent of anthocyanin coloration; H= Plant: height; NPP= Number of pod per plant; NSP= Number of seeds per pod; PL= Pod: length; PW= Pod: width; PV= Pod: villosity; SS= Dry seed: shape of median longitudinal section; 100SW= 100 seeds weight; SC= Seed: colour of seed coat; SH= Seed: pigmentation of the hilum.*

In the third quadrant, we find the 3 vectors of the length of the basal leaf, the villosity of the pod and the shape of the seed. In the fourth quadrant, we find the traits that show some correlation with those of the second quadrant (increasing of the second component, the first decreases, and vice versa), specifically the vectors of the length and width of the pod, of the maximum width position of the basal leaf, and the short vector of the closure of the flower.

#### *2.4.1.3. Clusters analysis and relations between the lines*

By a Cluster analysis have been identified 8 groups of accessions, indicated by the same number of colours and symbols, as it can be observed in the dendrogram in Figure 2.16.

The coloured map in the centre of the image is the representation of all the variables of phenotypic traits in the study population. Every shade indicates a different variable of the detected character.

Immediately catch the eye, in the coloured map, the nearby coloured columns, which are those relating to the traits related to the presence of tannins (anthocyanin colouration of the stem and of the flower stendard, spots on flower wings, pigmentation of the hilum) that allow to identify two large groups, two superclusters, one composed by the lines with the presence of tannins and the other, larger, from 54 accessions triple-white, with the absence of tannins.

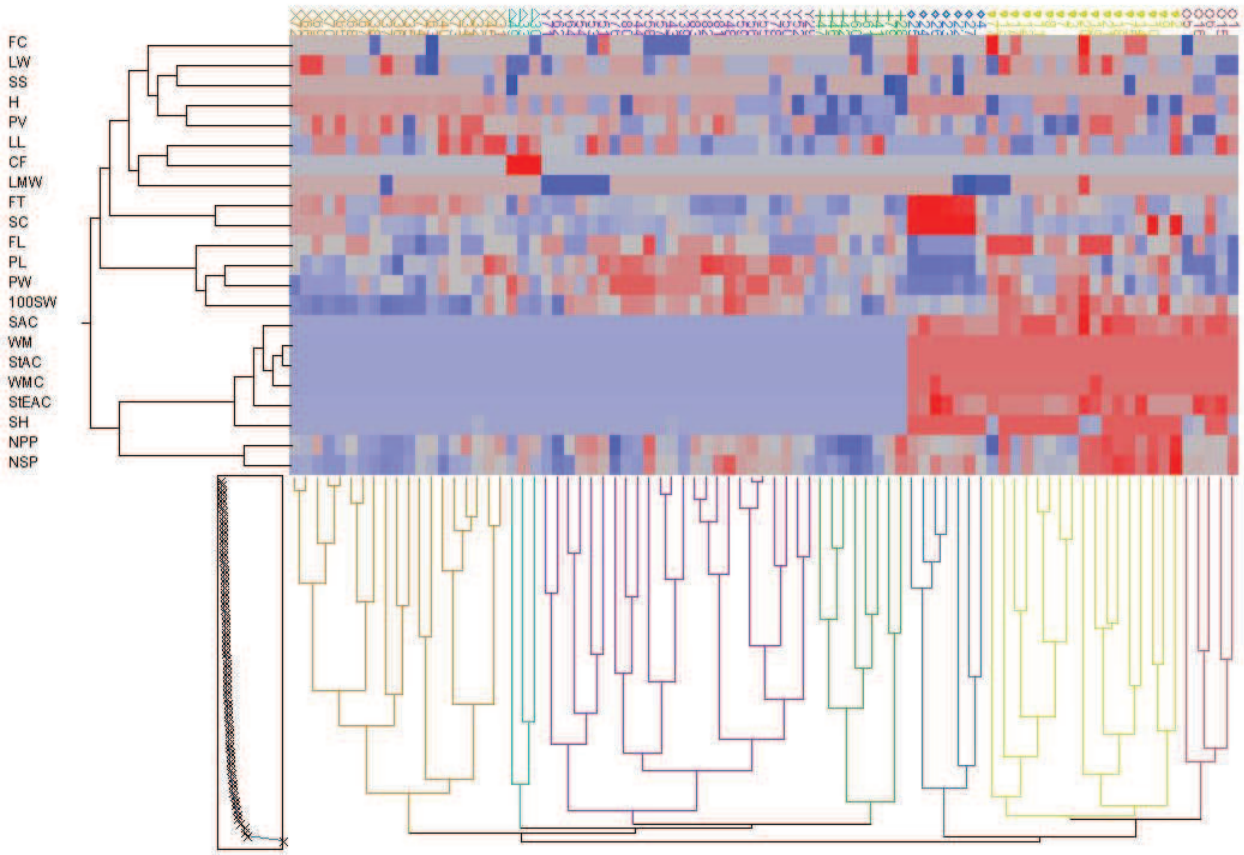


**Fig. 2.16. Double cluster analysis. To the right, a dendrogram on the similarity between the accessions and to the left, the list of lines, identified, per cluster, with a colour and a symbol. Below, the dendrogram on the correlation between characters.** FC= Foliage, intensity of green colour; FT= Time of flowering (50% of the plants with at least one flower opened); SAC= Stem: anthocyanin coloration; LL= Leaflet: length (basal pairs of leaflets at secondary node); LW= Leaflet: width (basal pairs of leaflets at secondary node); LMW= Leaflet: position of maximum width (basal pairs of leaflets at secondary node); FL= Flower: length; CF= Flower: closure; WM= Wings: melanin spot; WMC= Wings: colour of melanin spot; STAC= Stendard: anthocyanin coloration; STEAC= Stendard: extent of anthocyanin coloration; H= Plant: height; NPP= Number of pod per plant; NSP= Number of seeds per pod; PL= Pod: length; PW= Pod: width; PV= Pod: villosity; SS= Dry seed: shape of median longitudinal section; 100SW= 100 seeds weight; SC= Seed: colour of seed coat; SH= Seed: pigmentation of the hilum.

Within the supercluster of coloured lines, there are four clusters:

- *Red group (A), indicated by the circle symbol*: includes 5 accessions, IS1, IS5, IS6, IS15 and IS16. These lines have seed coat "white", number of pods and seeds per plant in the middle of the 83 accessions, and small pods.

- *Yellow group (B), indicated by the inverted triangle symbol*: includes 9 accessions, IS2, IS9, IS10, IS14, IS18, IS19, IS20, IS21 and IS75. These lines are distinguished from the other coloured lines, because they present a number of pods per plant and seeds per pod relatively high, and the pods of greater dimensions.

- *Teal group (C), indicated by the* Z *symbol*: includes the 8 accessions IS3, IS7, IS8, IS11, IS12, IS13, IS17 and IS77. These accessions present the number of pods per plant and seeds per pod the lowest of the super-cluster and long flowers.

- *Blue group (D), indicated by the rhombus symbol*: includes 7 lines, the accessions IS4, IS22, IS23, IS24, IS25, IS26 and IS27. These accessions are the lines with late flowering time, of 83 lines examined. Each has a short flower pods small, and black seed coat.

Within the supercluster of triple-white lines, there are other four clusters:

- *Green group (E), indicated by the plus symbol*: includes the lines IS28, IS41, IS42, IS46, IS47, IS60, IS61 and IS79. All the 8 lines have short plants, the number of pods per plant and seeds per pod the lowest of the super-clusters, and pods are tendentially hairless.

- Purple group (F), *indicated by the* Y *symbol:* is by far the largest cluster of the entire population, and includes 24 lines: IS29, IS39, IS43, IS44, IS49, IS50, IS51, IS52, IS53, IS54, IS55, IS56, IS57, IS58, IS59, IS62, IS64, IS71, IS76, IS78, IS81, IS82, IS83. It includes *V. faba* lines with greater 100 seeds weight of the super cluster of the white lines. Moreover, these accessions have number of pods per plant and seeds per pod relatively high, and, as those of the cluster E, have early flowering time.

- *Turquoise group (G), indicated by the triangle symbol:* includes accessions IS30, IS33 and IS49, the only 3 lines who have expressed all the three years considered, the trait of the closed flower.

- *Orange group (H), indicated by the* X *symbol:* the white accessions comprised in this cluster are IS31, IS32, IS34, IS35, IS36, IS37, IS38, IS40, IS45, IS63, IS65, IS66, IS67, IS68, IS69, IS70, IS72, IS73 and IS74. This group includes the 19 less

productive accessions, with number of pods per plant, number of seeds per pod, and 100 seeds weight, with very low values.

In figure 2.17, we can observe how the 8 groups of identified lines are arranged on the Cartesian plane with respect to the Principal Components.

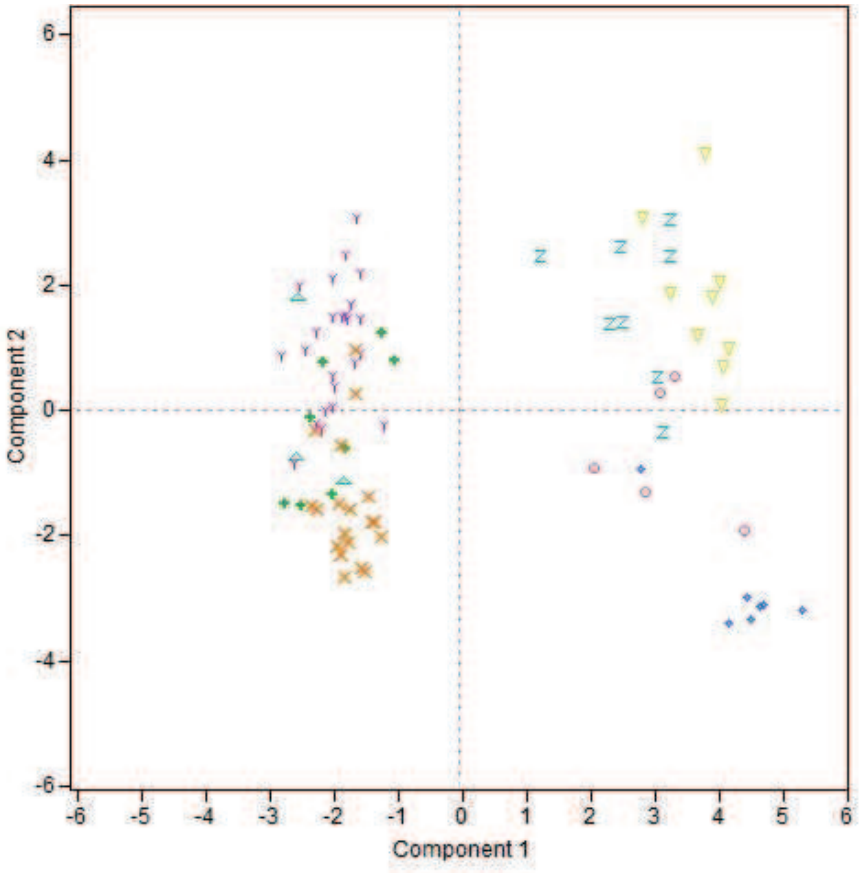


**Fig. 2.17. Result of Analysis PCA on the 83 accessions: behaviour of clusters of accessions, indicated by coloured symbols, in relation to the two Principal Components selected.**

We can make two observations. The first is that the two super clusters of white lines (in the left of the plot score) and coloured ones (right) are very well defined and distinct: this proves the fact that the Main Component one is constituted mainly by the traits related to the presence of tannins in the plant.

Moreover, it can be noticed that, despite the variability between the lines, there is still a certain genetic similarity among of the group of the white lines, which appear grouped together. This is surely due to the common origin of many accessions and continuous selection for same characteristics. The distance between the lines in the coloured accessions group, is to be charged, in addition to their diversified origin, and also to the discontinuous or not careful selection over the years.

### 2.4.2 Agronomic evaluation of the lines

The data obtained from the agronomic evaluation on plots conducted in the year 2011-2012 on the 83 lines examined are shown in Table 2.5.

The MSE, calculated on the controls, was found to be 0.21. The average production of the blocks, calculated on the averages of the productions of the controls of each block are respectively 2.04, 1.67 and 1.74 for the first, second and third block.

Through these results, we have identified: the lines whose yield differences are within the standard deviation between the blocks

(indicated by the symbol °), and the lines that have yield differences within the standard deviation within the block (indicated by the symbol *). The lines whose yields remains within the standard deviations are considered statistically equal

### 2.4.3 Choice of synthetic varieties

The lines selected for the creation of synthetic are the lines indicated in the table 2.6. The clusters indicated in the table, are those derived from the agronomic and phenotypic characterization.

Two different tests, in two years, were carried out in the field; we proceeded to test, in the year 2013-2014, 3 synthetic varieties, and in the year 2014-2015, 4 synthetic varieties. The composition of the Synthetics is shown in table 2.7. For the selection of the lines forming the synthetic varieties of the first group (IS-Sint1, IS-Sint2, IS-Sint3), we considered the clusters derived from the analysis of phenotypic data until 2013 (Cerquetti, 2013), which are shown in brackets.

As the table shows, the accessions that form the synthetic varieties, not in every combination belong to the same cluster, and priority was given, instead, to the belonging to different groups of Pedigree. We made this choice because, although the accessions are in different clusters, the inbred lines chosen are phenotypically very similar, and it is preferred a higher number of varieties tested by year.

**Tab. 2.5 Yield of the 83 lines of the population in the preliminary test on production (2011-2012).**

| Block | Line | Plant coloration | YIELD (t/ha) |
|---|---|---|---|
| 1 | **IS37** | Triple white | 1.82*° |
| 1 | **IS65** | Triple white | 1.72*° |
| 1 | **IS40** | Triple white | 1.63*° |
| 1 | **IS36** | Triple white | 1.62*° |
| 1 | **IS77** | Coloured | 1.62*° |
| 1 | **IS29** | Triple white | 1.62*° |
| 1 | **IS34** | Triple white | 1.57*° |
| 1 | **IS72** | Triple white | 1.54*° |
| 1 | **IS30** | Triple white | 1.45*° |
| 1 | **IS35** | Triple white | 1.33*° |
| 1 | **IS42** | Triple white | 1.3*° |
| 1 | **IS41** | Triple white | 1.29*° |
| 1 | **IS76** | Triple white | 1.24*° |
| 1 | **IS28** | Triple white | 1.22*° |
| 1 | **IS38** | Triple white | 1.21*° |
| 1 | **IS46** | Triple white | 1.19*° |
| 1 | **IS31** | Triple white | 1.09 |
| 1 | **IS32** | Triple white | 1.07 |
| 1 | **IS67** | Triple white | 1.04 |
| 1 | **IS74** | Triple white | 1.03 |
| 1 | **IS66** | Triple white | 1.03 |
| 1 | **IS33** | Triple white | 1.03 |
| 1 | **IS71** | Triple white | 1.02 |
| 1 | **IS69** | Triple white | 1.01 |
| 1 | **IS73** | Triple white | 1 |
| 1 | **IS68** | Triple white | 0.92 |
| 1 | **IS47** | Coloured | 0.83 |
| 1 | **IS70** | Triple white | 0.73 |
| **Average of tests block (1)** | | | **2.04** |

| Block | Line | Plant coloration | YIELD (t/ha) |
|---|---|---|---|
| 2 | **IS2** | Coloured | 1.51*° |
| 2 | **IS51** | Triple white | 1.46*° |
| 2 | **IS56** | Triple white | 1.37*° |
| 2 | **IS64** | Triple white | 1.28*° |
| 2 | **IS61** | Triple white | 1.25*° |
| 2 | **IS57** | Triple white | 1.22*° |
| 2 | **IS81** | Triple white | 1.19*° |
| 2 | **IS52** | Triple white | 1.19*° |
| 2 | **IS54** | Triple white | 1.18° |
| 2 | **IS62** | Triple white | 1.17° |
| 2 | **IS11** | Coloured | 1.17° |
| 2 | **IS82** | Triple white | 1.1° |
| 2 | **IS83** | Triple white | 1.08° |
| 2 | **IS80** | Triple white | 1.03° |
| 2 | **IS15** | Coloured | 1.02° |
| 2 | **IS59** | Triple white | 1.01° |
| 2 | **IS48** | Triple white | 0.94° |
| 2 | **IS60** | Triple white | 0.92° |
| 2 | **IS58** | Triple white | 0.88° |
| 2 | **IS55** | Triple white | 0.76 |
| 2 | **IS50** | Triple white | 0.73 |
| 2 | **IS49** | Triple white | 0.68 |
| 2 | **IS53** | Triple white | 0.68 |
| 2 | **IS63** | Triple white | 0.65 |
| 2 | **IS16** | Coloured | 0.59 |
| 2 | **IS79** | Triple white | 0.58 |
| 2 | **IS78** | Triple white | 0.57 |
| C | **IS10** | | 0.48 |
| **Average of tests. block (2)** | | | **1.67** |
| 3 | **RIEMP.** | Coloured | 2.35*° |
| 3 | **IS23** | Coloured | 1.95*° |

| Block | Line | Plant coloration | YIELD (t/ha) |
|---|---|---|---|
| 3 | **IS19** | Coloured | 1.61* |
| 3 | **IS18** | Coloured | 1.5* |
| 3 | **IS24** | Coloured | 1.49* |
| 3 | **IS17** | Coloured | 1.48* |
| 3 | **IS13** | Coloured | 1.48* |
| 3 | **IS25** | Coloured | 1.47* |
| 3 | **IS6** | Coloured | 1.4* |
| 3 | **IS7** | Coloured | 1.4* |
| 3 | **IS14** | Coloured | 1.38* |
| 3 | **IS9** | Coloured | 1.13 |
| 3 | **IS27** | Coloured | 1.11 |
| 3 | **IS21** | Coloured | 1.05 |
| 3 | **IS22** | Coloured | 1.05 |
| 3 | **IS44** | Triple white | 1 |
| 3 | **IS20** | Coloured | 0.98 |
| 3 | **IS1** | Coloured | 0.98 |
| 3 | **IS45** | Triple white | 0.95 |
| 3 | **IS12** | Coloured | 0.91 |
| 3 | **IS39** | Triple white | 0.91 |
| 3 | **IS8** | Coloured | 0.91 |
| 3 | **IS43** | Triple white | 0.87 |
| 3 | **IS26** | Coloured | 0.77 |
| 3 | **IS5** | Coloured | 0.71 |
| 3 | **IS75** | Coloured | 0.59 |
| 3 | **IS4** | Coloured | 0.46 |
| 3 | **IS3** | Coloured | 0.44 |
| **Average of tests block (3)** | | | **1.74** |
| 3 | **TEST1** | Coloured | 3.02 |
| 1 | **TEST2** | Coloured | 1.98 |
| 2 | **TEST1** | Coloured | 1.93 |
| 3 | **TEST2** | Coloured | 1.74 |

| Block | Line | Plant coloration | YIELD (t/ha) |
|---|---|---|---|
| 1 | **TEST1** | Coloured | 2.10 |
| 2 | **TEST2** | Coloured | 1.40 |
| **MSE tests** | | | **0.21** |
| **Average of tests** | | | **1.83** |
| **DS within block (3)** | | | **0.65** |
| **DS among blocks** | | | **0.63** |

**Tab. 2.6 Lines selected for the creation of synthetics. The clusters are those obtained frome the phenotypic and agronomic evaluation. See paragraph 2.4.1.3.**

| Pedigree Group | Code | Cluster* |
|---|---|---|
| G1 – 1 | IS28 | E |
| G1 – 3 | IS31 | H |
| G1 – 2 | IS42 | E |
| G1 – 2 | IS46 | E |
| G2 | IS67 | H |
| G3 – 2 | IS79 | E |
| G3 – 1 | IS80 | F |
| G3 – 1 | IS81 | F |
| G4 – 1 | IS48 | F |
| G4 – 2 | IS51 | F |
| G4 – 2 | IS52 | F |
| G4 – 3 | IS54 | F |
| G4 – 3 | IS56 | F |
| G4 – 5 | IS58 | F |
| G4 – 4 | IS59 | F |

**Tab. 2.7: The combinations of inbred lines, of which the synthetic varieties are composed.**
(*): in brackets are indicated the clusters obtained in the first phenotypic characterization of the material, and used in 2013 for the selection of accessions forming the synthetic varieties IS-Sint1, IS-Sint2, IS-Sint3 (Cerquetti, 2013).

| Synthetic Line | Pedigree Group | Code | Cluster (*) |
|---|---|---|---|
| IS-Sint1 | G1 – 1 | IS28 | E (E) |
| | G4 – 3 | IS56 | F (E) |
| | G4 – 4 | IS59 | F (E) |
| IS-Sint2 | G4 – 2 | IS51 | F (F) |
| | G4 – 3 | IS54 | F (F) |
| | G4 – 5 | IS58 | F (F) |
| IS-Sint3 | G4 – 1 | IS48 | F (E) |
| | G3 – 2 | IS79 | E (E) |
| | G3 – 1 | IS81 | F (E) |
| IS-Sint4 | G1 – 1 | IS28 | E |
| | G1 – 2 | IS46 | E |
| IS-Sint5 | G1 – 3 | IS31 | H |
| | G4 – 1 | IS48 | F |
| | G4 – 3 | IS56 | F |
| | G3 – 1 | IS81 | F |
| IS-Sint6 | G4 – 2 | IS52 | F |
| | G2 | IS67 | H |
| | G3 – 1 | IS80 | F |
| IS-Sint7 | G1 – 2 | IS42 | E |
| | G4 – 2 | IS51 | F |
| | G4 – 3 | IS54 | F |

### *2.4.3.1 Field tests for the assessment of the synthetic's yield*

*2.5.3.1.1 Year 2013-2014*

Three synthetic lines were tested, IS-Sint1, IS-Sint2 and IS-Sint3, together with 5 coloured inbred lines and 4 commercial varieties used as test. The data collected are shown in table 2.8.

**Tab. 2.8. Results of the 2013-2014 field trial. The numbers in brackets refer to the block number. Synthetic lines are coloured in red and inbred lines in blue.**

| Block | Variety name | Yield (t/ha) | 100 seeds Weight (g) | Date of flowering (days from March 1st) | Density (0-9) | Plant Height (cm) |
|---|---|---|---|---|---|---|
| 1 | **RUMBO** | **3,31** | 45,60 | 41 | 8 | 110 |
| 1 | **RUMBO** | **2,83** | 46,04 | 41 | 8 | 125 |
| 1 | **SCURO TORRELAMA** | **2,60** | 38,30 | 41 | 8 | 120 |
| 1 | **SCURO TORRELAMA** | **2,41** | 38,42 | 41 | 7 | 115 |
| 1 | **IS9** | **2,04** | 64,12 | 30 | 9 | 118 |
| 1 | **COLLAMENO** | **1,94** | 43,00 | 44 | 8 | 115 |
| 1 | **CHIARO TORRELAMA** | **1,85** | 40,84 | 44 | 8 | 121 |
| 1 | **COLLAMENO** | **1,81** | 39,24 | 44 | 8 | 110 |
| 1 | **CHIARO TORRELAMA** | **1,52** | 42,12 | 43 | 9 | 120 |
| 1 | **IS107** | **1,43** | 83,04 | 32 | 8 | 100 |
| 1 | **IS-Sint2** | **1,13** | 77,34 | 30 | 9 | 53 |
| 1 | **IS-Sint1** | **0,88** | 72,60 | 33 | 9 | 70 |
| Average of the block (1) | | **1,98** | 52,56 | 38,67 | 8,25 | 106,42 |
| Min value | | **0,88** | 38,30 | 30,00 | 7,00 | 53,00 |
| Max value | | **3,31** | 83,04 | 44,00 | 9,00 | 125,00 |
| Average of the tests (1) | | **2,28** | 41,70 | 42,38 | 8,00 | 117,00 |
| SD within Block (1) | | **0,57** | 3,25 | 1,41 | 0,50 | 5,10 |

| Block | Variety name | Yield (t/ha) | 100 seeds Weigh t (g) | Date of flowerin g (days from March 1st) | Density (0-9) | Plant Height (cm) |
|---|---|---|---|---|---|---|
| 2 | **RUMBO** | **3,03** | 43,44 | 40 | 8 | 120 |
| 2 | **IS18** | **3,02** | 61,72 | 32 | 9 | 110 |
| 2 | **SCURO TORRELAMA** | **2,91** | 37,20 | 41 | 8 | 110 |
| 2 | **SCURO TORRELAMA** | **2,61** | 36,18 | 40 | 8 | 125 |
| 2 | **RUMBO** | **2,24** | 44,10 | 41 | 8 | 115 |
| 2 | **IS15** | **2,22** | 70,04 | 31 | 9 | 98 |
| 2 | **CHIARO TORRELAMA** | **2,10** | 40,32 | 45 | 8 | 115 |
| 2 | **CHIARO TORRELAMA** | **2,00** | 37,58 | 44 | 8 | 112 |
| 2 | **COLLAMENO** | **1,92** | 35,72 | 45 | 8 | 110 |
| 2 | **COLLAMENO** | **1,85** | 33,90 | 45 | 8 | 118 |
| 2 | **IS25** | **0,97** | 27,74 | 42 | 9 | 107 |
| 2 | **IS-Sint3** | **0,43** | 87,50 | 33 | 9 | 90 |
| Average of the block (2) | | **2,11** | 46,29 | 39,92 | 8,33 | 110,83 |
| Min value | | **0,43** | 27,74 | 31,00 | 8,00 | 90,00 |
| Max value | | **3,03** | 87,50 | 45,00 | 9,00 | 125,00 |
| Average of the tests (2) | | **2,33** | 38,56 | 42,63 | 8,00 | 115,63 |
| SD within Block (2) | | **0,43** | 3,80 | 2,18 | 0,00 | 4,92 |
| Average of the tests | | **2,31** | 40,13 | 42,50 | 8,00 | 116,31 |
| MSE (test) | | **0,26** | **12,52** | **3,38** | **1,12** | **25,09** |
| SD among Blocks | | **0,51** | **3,54** | **1,84** | **0,35** | **5,01** |
| Average (TOTAL) | | **2,04** | 49,42 | 39,29 | 8,29 | 108,63 |
| Min value | | **0,43** | 27,74 | 30,00 | 7,00 | 53,00 |
| Max value | | **3,31** | 87,50 | 45,00 | 9,00 | 125,00 |

We focus mainly the results on the yield, which is what interests us most: The highest value observed in the test is 3.03 t/ha, and the lowest

value is 0.43 t/ha. The average value is 2.04 t/ha. The average production of the blocks, are respectively 1.58 t/ha and 2.11 t/ha for the first and the second block. The average production of the controls for the first block is 2.28 t/ha, for the second block is 2.33 t/ha, and the general mean of the tests varieties is 2.31 t/ha.

The MSE (Error Mean Square), calculated on control varieties (tests) was found to be 0.21, the standard deviation (SD) for the block 1 was found to be 0.57, 0.43 for the block 2, and the SD among the blocks is 0.51.

As can be easily see, the yield of the three synthetics, (respectively 0.88, 1.13, 0.43), at generation Syn1, is well below the overall averages and the averages of the controls.

However, the IS-Sint2 is found to be the most promising line; in one case (in the first block), was found to have a yield statistically comparable to that of the variety "Chiaro di Torrelama". The synthetic lines have a 100 seeds weight among the highest of the tested lines, between 72.6 g and 86.5 g; the average weight of 100 seeds, for the entire test is 49.42 g, and the average weight of the control varieties is 40.13.

On the other hand, the results of certain of the inbred lines in testing, in particular IS18, who had a production analogous to that of the more productive variety of the test, “Rumbo”, is interesting.

*2.5.3.1.2 Year 2014-2015*

Four synthetic lines were tested, IS-Sint4, IS-Sint5, IS-Sint6 and IS-Sint7, together with 5 coloured inbred lines and 4 commercial varieties used as test.

The data collected are shown in table 2.9.

**Tab. 2.9. Results of the 2014-2015 field trial. The numbers in brackets refer to the block number. Synthetic lines are coloured in red and inbred lines in blue.**

| Block | Variety name | Yield (t/ha) | 100 seeds Weight (g) | Date of flowering (days from March 1st) | Density (0-9) | Plant Height (cm) |
|---|---|---|---|---|---|---|
| 1 | **PROTHABAT 69** | **3,22** | 59,11 | 47 | 8 | 75 |
| 1 | **CHIARO TORRELAMA** | **2,92** | 42,75 | 50 | 8 | 80 |
| 1 | **RUMBO** | **2,88** | 44,80 | 48 | 8 | 90 |
| 1 | **RUMBO** | **2,73** | 44,35 | 50 | 7 | 80 |
| 1 | **PROTHABAT 69** | **2,67** | 60,06 | 47 | 8 | 80 |
| 1 | **SCURO TORRELAMA** | **2,31** | 38,94 | 49 | 8 | 75 |
| 1 | **SCURO TORRELAMA** | **2,30** | 34,72 | 48 | 8 | 90 |
| 1 | **CHIARO TORRELAMA** | **2,20** | 38,59 | 50 | 6 | 90 |
| 1 | **IS9** | **1,77** | 63,82 | 44 | 7 | 70 |
| 1 | **IS18** | **1,65** | 71,48 | 44 | 5 | 70 |
| 1 | **IS-Sint6** | **1,53** | 63,63 | 41 | 7 | 85 |
| 1 | **IS-Sint4** | **1,34** | 59,09 | 40 | 7 | 90 |
| Average of the block (1) | | **2,29** | 51,78 | 46,50 | 7,25 | 81,25 |
| Min value | | **1,34** | 34,72 | 40,00 | 5,00 | 70,00 |
| Max value | | **3,22** | 71,48 | 50,00 | 8,00 | 90,00 |
| Average of the tests (1) | | **2,65** | 45,41 | 17,63 | 7,63 | 82,50 |
| SD within Block (1) | | **0,34** | 9,09 | 1,23 | 0,70 | 7,91 |

| Block | Variety name | Yield (t/ha) | 100 seeds Weight (g) | Date of flowering (days from March 1st) | Density (0-9) | Plant Height (cm) |
|---|---|---|---|---|---|---|
| 2 | **CHIARO TORRELAMA** | **3,28** | 46,85 | 51 | 7 | 95 |
| 2 | **SCURO TORRELAMA** | **3,11** | 39,76 | 49 | 8 | 95 |
| 2 | **CHIARO TORRELAMA** | **2,91** | 41,31 | 50 | 7 | 105 |
| 2 | **SCURO TORRELAMA** | **2,90** | 38,37 | 49 | 8 | 95 |
| 2 | **RUMBO** | **2,82** | 44,86 | 49 | 8 | 100 |
| 2 | **PROTHABAT 69** | **2,64** | 71,13 | 48 | 8 | 80 |
| 2 | **RUMBO** | **2,43** | 47,86 | 50 | 8 | 90 |
| 2 | **PROTHABAT 69** | **2,36** | 72,44 | 46 | 7 | 80 |
| 2 | **IS96** | **1,88** | 57,90 | 48 | 7 | 80 |
| 2 | **IS-Sint5** | **1,50** | 102,20 | 40 | 7 | 110 |
| 2 | **IS138** | **1,49** | 74,55 | 45 | 7 | 65 |
| 2 | **IS-Sint-7** | **1,47** | 75,00 | 40 | 8 | 100 |
| **Average of the block (2)** | | **2,40** | 59,35 | 47,08 | 7,50 | 91,25 |
| **Min value** | | **1,47** | 38,37 | 40,00 | 7,00 | 65,00 |
| **Max value** | | **3,28** | 102,20 | 51,00 | 8,00 | 110,00 |
| **Average of the tests (2)** | | **2,81** | 50,32 | 18,00 | 7,63 | 92,50 |
| **SD within Block (2)** | | **0,31** | 13,01 | 1,43 | 0,48 | 9,68 |
| **Average of the tests** | | 2,73 | 47,87 | 48,81 | 7,63 | 87,50 |
| MSE **(test)** | | **0,11** | **125,98** | **1,78** | **0,36** | **78,13** |
| **SD among Blocks** | | **0,32** | **11,22** | **1,33** | **0,60** | **8,84** |
| **Average (TOTAL)** | | **2,35** | 55,57 | 15,79 | 7,38 | 86,25 |
| **Min value** | | **1,47** | 38,37 | 40,00 | 7,00 | 65,00 |
| **Max value** | | **3,28** | 102,20 | 51,00 | 8,00 | 110,00 |

We focus the data relative to the yield. The highest value observed in the test is 3.28 t/ha, and the lowest value is 1.47 t/ha.

The average value is 2.35 t/ha. The average production of the blocks, are respectively 2.29 t/ha and 2.40 t/ha for the first and the second block. The average production of the controls for the first block is 2.65 t/ha, for the second block is 2.81 t/ha, and the general mean of the tests varieties is 2.73 t/ha.

The MSE (Error Mean Square), calculated on control varieties (tests) was found to be 0.11, the standard deviation (SD) for the block 1 was found to be 0.34, 0.31 for the block 2, and the SD among the blocks is 0.32.

Also in this case, we observe the data relative to the yield of the synthetic tested significantly lower of the overall averages and the averages of the controls. The yield of the four synthetic lines resulted to be (at generation Syn1), 1.53, 15.0, 14.7 and 14.3 t/ha for respectively IS-Sint6, IS-Sint5, IS-Sint7 and IS-Sint4.

The productions of the 4 tested synthetic lines are statistically analogous, although there are differences in the size of the seed; the synthetic lines have 100 seeds weighing respectively 102.2 g (IS-Sint5), 75 g (IS-Sint7), 63.6 g (IS-Sint6) and 59,1 g (IS-Sint4).

The average weight of 100 seeds, for the entire test is 55.57 g, and the average weight of the control varieties is 47.87 g.

The production of coloured inbred lines is comparable to those of the synthetic varieties, and statistically lower than those of controls.

## 2.5 Discussion and Conclusions

The statistical analysis of the data of phenotypic characterization has shown the presence, within the starting population, of two large groups of greatly differentiated lines: one formed by accessions completely free of tannins, triple white, very stable, and one of coloured lines of *Vicia faba major*, *minor* and *equina*, very different in morphology and origins.

Within the second group, it can be observed greater variability. We can find very different genotypes among them, as well as for traits related to anthocyanin coloration, even for those morphological and yield.

The group of the triple-white, is essentially very stable, even if still comprises in its interior, a certain variability, for the morphological traits and production. The genetic variability between and within groups can be used for future breeding projects, with the aim of obtaining new varieties for different commercial purposes.

The observed correlation between traits is quite high, so that 48% of the variability is explained by only two components. The traits that appear as a solidly related group are those linked to the presence of tannins in the plant: anthocyanin colouration of the stem, presence and colour of melanin spots on flower's wings, colour and extent of anthocyanin colouration of the standard, and hilum black pigmentation. Two lines (IS19 and IS20), however, showed no correlation between these traits

and the hilum colour. A trait that is not correlated with the other, but interesting from the point of view of breeding, is the closure of the flower, found in only 3 lines of white flower: two F11 *V. faba spp. major* line, with tightly closed flower (IS30, IS33) and one *V. faba spp. minor* F7 line, with flower less closed (IS49). This feature is useful in the programs of improvement for the stability of the line, since it forces *V. faba* to self-pollination, and is especially useful in the case of the white-lines. White flower colour, together with tannin-free seed coat, is determined by two complementary zero-tannin genes, zt1 and zt2 (Picard 1967; Crofts *et al.* 1980), both of which have been mapped in faba bean (zt1, Gutierrez *et al.* 2007; zt2, Gutierrez *et al*. 2008). Colourless hilum is controlled by a single recessive gene (Sjödin 1971; Gutierrez *et al*., 2006) that is about 10 cM from low vicine–convicine (VC) content (Gutierrez *et al.,* 2006; Crépon *et al.,* 2010). All the triple-white traits are determined by recessive genes. Also, the genes that regulates the colouring of the stipules of faba bean leaves appears to be recessive, although it is not necessarily related to the traits of the Triple -white, these last related to the absence of tannins (Khazaei *et al*., 2014).

The test on plots, conducted with the Augmented Block design scheme, led to the identification of groups of homogeneous lines, likely to be more productive than the other, with similar productions to commercial varieties.

All the analyses carried up-to-date, have allowed us to observe that, once the traits of interest fixed (absence of tannins), there is still a certain variability within the population. It can be potentially exploited for the establishment of new varieties, for different classes and commercial purposes, using also the genetic resources of the precocity and the closed flower. This ensures a high degree of self-pollination, important feature providing an acceptable production in case of lack of pollinators.

The phenotypic and agronomic evaluation were used to identify the best genotypes, which have been employed for the establishment of new synthetic varieties. The goal was to obtain a commercial line characterized by having yield stability, a good precocity and absence of anti-nutritional factors. Fifteen of these genotypes, the most productive, phenotypically similar and (according to the historical data derived by pedigree method) genotypically different, have been combined to form 7 synthetic varieties.

The decision to create synthetic lines of faba bean, instead of marketing inbred lines triple-white (white flower, white seed coat and white hilum, all recessives traits, correlated with a good nutritional value), comes from the commercial need to increase and stabilize the yield of these kind of lines, while keeping homogeneous the phenotype. So, we selected lines that are phenotypically very similar.

The choice of the lines composing the synthetic varieties, has been driven, for technical reasons, mainly on the availability of seed and not having available genomic data, from historical data (pedigree). We have chosen to follow this policy because, from a business perspective, it is preferable to have as many tests as possible and advance with the material reproduction, in order to gain time to get to the commercialization of the new variety.

The results of the two agronomic trials (2013-2014 and 2014-2015) have shown that synthetic lines examined, at the Syn1 generation, have productions statistically much lower than the commercial varieties productions used as a test. This fact is not surprising and is not indicative of the actual value of the Synthetic, as at the Syn1 generation there is still no a heterotic effect of polycross. It is important to keep in mind the fact that the data of yield is preliminary, and is not indicative of the actual value of the synthetic lines in advanced generations of polycrossing. However, it gives us a preliminary indication on agronomic performances, and allows us to discard the lines that are not phenotypically good, like IS-Sint1 and IS-Sint3, that it was decided to stop multiplying.

Another reason of the low yield of the synthetic lines, is the 100 seeds weight, among the highest of the tested lines, a trait that has correlation with production (Duc, 1997).

An important data, which has not been possible to collect in these two tests, is the number of pods and seeds per plant (NPP, NSP), which should be analysed for correlation with the weight of 100 seeds. In fact, a higher weight of 100 seeds, is generally negatively correlated with high NPP and NSP (Kambal, 1969), which is also confirmed by the results of the phenotypic characterization.

Synthetic lines with acceptable productions (IS-Sint2; IS-Sint4; IS-Sint5; IS-Sint6; IS-Sint7), will be multiplied and re-tested for agronomic value. The most productive lines, maintaining the phenotypic characteristics required, will become commercial varieties after registration.

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CHAPTER 3

# Evaluation of inbred lines triple-white, for resistance to Rust (*Uromyces viciae-fabae*) and Chocolate spot (*Botrytis fabae*)

## 3.1 Introduction: Breeding for biotic stresses in faba bean.

The faba bean (*Vicia faba* L.) is a crop grown worldwide under a range of climatic conditions from temperate to subtropical. It hosts a wide variety of regional, native and exotic cosmopolitan insect pests, fungal pathogens, viruses and parasitic weeds (in particular, *Orobanche spp.*) that may cause severe diseases in faba bean crops (Stoddard *et al*., 2010, Duc *et al*., 2015). Depending on the environmental conditions of cultivation, the issues can be of different kind.

There are three types of cultivated faba bean lines: Mediterranean-adapted lines, cool-temperate winter adapted lines, and spring cultivated lines. These varieties are exposed to different pests and diseases at different times of their growth cycles. Mediterranean and winter-type faba beans are sown in autumn, in conditions of abundant but declining pest activity, that then remains relatively low until temperatures increase in early spring. These two types of faba bean optimize use of resources but are exposed to pests and diseases for a longer time. Spring-sown beans, on the other hand, are directly exposed

to pests and diseases from emergence, and often a different suite of parasites and pathogens is involved.

In the Mediterranean basin, broomrapes are the major limitation to growing faba bean. The main species of these root parasites are *Orobanche crenata* Forsk., *Orobanche foetida* Poir. and *Phelipanche aegyptiaca*. The most important and diffused for faba bean is *Orobanche crenata* (Duc *et al*., 2015). The resistance to this broomrape is generally quantitative (Díaz-Ruiz *et al*. 2010).

To reduce the negative effects of the disease on yield, and also the use of pesticides, good farming practices with the use of accessions resistant or tolerant to the different pathogens must be combined. The greatest work of breeders is precisely the search for new sources of tolerance to pathogens, that must be transferred into cultivated varieties, in order to reduce the risks linked to the resistance.

### 3.1.1. Fungal diseases in faba bean.

Chocolate spot, caused by *Botrytis fabae* Sard. (figure 3.1), is present in all regions where faba bean is grown. Due to its expression depends particularly strongly on environmental conditions, it is one of the most difficult diseases to handle in a breeding programme (Duc *et al*., 2015). An attack of this pathogen considerably lowers the yield in faba beans planted in autumn (Duc *et al*., 1997). When conditions are just right for the fungus for about 48 h (damp leaves, high atmospheric humidity,

temperatures close to 20 °C), it grows rapidly on the leaves, until the whole plant is dead. A good number of potential sources of resistance (quantitative) to *B. fabae* have been identified (Sillero *et al*., 2010); the sources used by ICARDA breeding programmes are mainly from South America (Tivoli *et al*., 2006).

**Fig. 3.1. Plant affected by Chocolate spot (*Botrytis fabae*)**

Ascochyta blight, caused by *Ascochyta fabae* Speg. (teleomorph *Didymella fabae* Jellis and Punithalingam), is a common disease that causes up to 90% yield losses in susceptible cultivars when

environmental conditions are favourable for disease development (Hanounik, 1980). It is prevalent on autumn-sown faba bean, including Mediterranean climates (Duc *et al*., 2015). The symptoms are necrotic and depressed spots on the pods, blackish, which extend to the seeds that are developing (figure 3.2).

Resistance to this pathogen was found in the line 29H, with an effect of dominance and polygenic determinism (Maurin, 1989), that is one of the most resistant to the pathogen accessions ever described (Atienza *et al*., 2016). Breeding for resistance led to the identification of major and minor genes (Kohpina *et al*. 2000) and quantitative trait loci (QTLs; Román *et al*. 2003; Avila *et al*. 2004) related to resistance. Some accessions have shown stable resistance across environments, whereas others were more resistant in either the Mediterranean or continental environment (Rubiales *et al*., 2012).

The faba bean Rust (*Uromyces fabae-viciae*) (Figure 3.3) is responsible for leaf loss and declining production in countries where the heat comes at the end of flowering, ie the countries of the Mediterranean, Eastern Europe and central Canada. During the last century, several sources of resistance have been found (Sillero *et al*., 2010; Sillero *et al*. 2006). Some lines have been identified and with incomplete resistance. Additionally, it has also been identified a typology resistance called "hypersensitive resistance", where the necrotic spots appear later and

**Fig. 3.2 Symptoms of Anthracnosis (*Ascochyta fabae*) on faba bean seeds.**

**Fig. 3.3 Plant affected by Rust (*Uromyces viciae-fabae*)**

are less problematic: there is a reduction in symptoms of disease rather than a resistance (Sillero *et al*., 2011).

Combined disease resistance is obviously valuable, and it has been possible to select resistances to chocolate spot disease and rust disease together (Villegas-Fernández *et al*., 2011).

The root rots may develop in wet and warm soil situations. These are mainly attributed to *Fusarium oxysporum*, *F. solani*, *F. culmorum* and *F. avenaceum*. Tannin-free lines tend to be more susceptible (Pascual-Villalobos and Jellis, 1990; Helsper *et al*., 1994; Kantar *et al*., 1996). This suggests that tannins may have fungicidal properties which protect seeds and seedlings during germination and emergence, but also in white fava beans resistant lines were found (EU CAMAR program). Seed treatment is an effective additional protection, but it can lead to the increase of resistance (Duc *et al*., 1997).

Downy mildew, caused by *Peronospora viciae* f. sp. *fabae* (Berk.), is usually more problematic in north western Europe. Soil-borne oospores cause systemic infection of emerging plants, producing wind dispersed conidia which give rise to cycles of secondary or localised infection. Epidemics can develop rapidly, and yield losses are highest when infection increases during early flowering or even before flowering

(Sillero *et al*., 2010). Resistance to downy mildew has been identified in the UK germplasm (Thomas and Kenyon 2004).

#### *3.1.1.1. Chocolate spot*

Chocolate spot is especially damaging in humid areas, where is the cause of heavy reductions in yields in places such as the Maghreb, Southern China, Egypt, UK or France (Bouhassan *et al*., 2004; Tivoli *et al*., 2006).

The first symptoms are discrete dark-brown spots surrounded by an orange-brown ring on leaves, flowers and stems. With temperatures around 15–22 °C, and a high relative humidity (>80%), the disease can become very aggressive, with necrosis spreads rapidly, defoliation and death of the plant, sometimes within a couple of days (Harrison, 1988). The most important damage usually occurs in flowering, which usually corresponds to the period of year when the environmental conditions are more favourable to the disease. In this period, the decaying flower petals are available for growth of the fungus, that spreads from the flower to developing pods, and can led to a strong yield reductions.
Spores of *Botrytis fabae* are produced by conidiophores that are easily visible with the naked eye in senescent leaves of diseased plants; the conidia are transported by air, so the disease can spread quickly. The fungus survives as mycelium or sclerotia in crop residues, until

temperature and humidity are again favourable to the development of the conidiophores (Stoddard *et al*., 2010).

There is not a comprehensive description of races of *B. fabae*, although differences in the virulence of isolates have been reported (Hutson and Mansfield, 1980), and the existence of races has been proposed (Hanounik and Maliha, 1986).

The major constraint for faba bean breeding for chocolate spot resistance is the lack of good sources of resistance. Total resistance has not been reported yet, and only incomplete resistance is being used.

A certain number of sources of resistance has been reported in the last years (Sillero *et al*., 2010) but only low levels of resistance are actually available in faba bean commercial varieties (Villegas-Fernández *et al*., 2012). Resistant germplasm originates mainly from the Andean region of Colombia and Ecuador (Bond *et al*., 1994; Sillero *et al*., 2010).

#### *3.1.1.2 Rust*

Faba bean rust, is a major disease in the Middle East, North Africa and parts of Australia, but has worldwide distribution.

*U. viciae-fabae sensu lato* is a species complex. Host specialized isolates that cannot infect *V. faba* have been reported by Emeran *et al*. (2005; 2008), suggesting that the species *U. viciae-fabae* may be subdivided into at least 3 groups with differential pathogenicity respectively to faba bean, vetch or lentil.

Usually the development of the disease begins late in the season, when the pod filling has already started, so yield components are little affected by the infection, with losses ranging from 5 to 20%, but have been reported cases of early infection that can led to important yield losses, even 70% (Liang, 1986, Rashid and Bernier, 1991).

*Uromyces viciae-fabae* is autoecious, and complete its life cycle on host plants (Stoddard *et al*., 2010).

The symptoms are visible as oval, brown-coloured uredial pustules, up to 1 mm in diameter. Rust affects all the above-ground parts of faba bean, and the pustules can develop on both surfaces of leaflets, branches, stems and pods.

Cloudy weather with high humidity and 17–22 °C favours development of the disease. (Stoddard *et al*., 2010).

Several sources of resistance against *U. viciae-fabae* have been reported in faba bean in the last decades. There are two different type of resistance: the incomplete resistance is most described (Rashid and Bernier, 1984, 1991; Khalil *et al*., 1985; Polignano *et al*., 1990; Sillero *et al*., 2000; Herath *et al*., 2001), and the hypersensitive resistance, that has only recently been identified by Sillero *et al*., (2000). In the case of the type hypersensitive response, the plant reacts to the infection of the pathogen killing the cells attacked by the fungus, resulting in a necrotic reaction. In this way, the pathogen is confined and does not have the

possibility to expand, resulting in a reduction of the infection type rather than complete resistance (Sillero *et al*., 2010).

## 3.2 Objective of the work

The aim of the work is to characterize under controlled conditions a selection of triple-white inbred lines and synthetic lines for their response to Rust and Chocolate spot, focusing on the identification of potential sources of resistance.

## 3.3 Materials and Methods

### 3.3.1 Plant materials and experimental design

Plant materials used for the tests, is a selection of triple-white (TW: white flower, white seed coat and white hilum) accessions, triple-white synthetics, breeding lines and commercial varieties, for a total of 17 *V. faba* accessions, plus 3 controls, presented in Table 3.1.

The triple-white accessions derive from a breeding program ISEA-ENEA for the improvement of Mediterranean faba bean (Bozzini *and* Chiaretti, 1997, 1999), whose phenotypic characterization was discussed in Chapter 2. The synthetics, have been assembled, from the abovementioned inbred lines, in the context of an improvement program by ISEA-ENEA for obtaining triple-white commercial varieties (see paragraph 2.4.5).

The lines were seeded in plastic pots filled with soil, one seed per pot, and placed in growth chamber at temperature 20 ° C and controlled photoperiod (12 hours of light and 12 of dark). Two independent experiments were carried out for each disease (i.e. two experiments for chocolate spot and another two for rust), with 3 replications per accession. Reached the fourth leaf stage, we proceeded with the inoculation.

All experiments were conducted at the IAS (Institute of Susteinable Agriculture) - CSIC of Córdoba, Spain.

**Tab 3.1. Plant 117 material. In red, the varieties used as control. In blue, the varieties used as control tests.**

| | Code | Line | Description |
|---|---|---|---|
| 1 | **IS18** | IS18 | *Inbred* |
| 2 | **IS28** | IS28 | *TW Inbred* |
| 3 | **IS42** | IS42 | *TW Inbred* |
| 4 | **IS46** | IS46 | *TW Inbred* |
| 5 | **IS48** | IS48 | *TW Inbred* |
| 6 | **IS54** | IS54 | *TW Inbred* |
| 7 | **IS56** | IS56 | *TW Inbred* |
| 8 | **IS80** | IS80 | *TW Inbred* |
| 9 | **IS89** | IS89 | *Inbred* |
| 10 | **IS156** | IS156 | *TW Inbred* |
| 11 | **IS-S4** | IS-Sint4 | *TW Synthetic* |
| 12 | **IS-S5** | IS-Sint5 | *TW Synthetic* |
| 13 | **IS-S6** | IS-Sint6 | *TW Synthetic* |
| 14 | **IS-S7** | IS-Sint7 | *TW Synthetic* |
| 15 | **RUMBO** | RUMBO | *Commercial Variety* |
| 16 | **STL** | Scuro di Torrelama | *Commercial Variety* |
| 17 | **CTL** | Chiaro di Torrelama | *Commercial Variety* |
| T | **BK** | Baraka | *Variety susceptible to Rust and Chocolate Spot* |
| T | **MARIMBA** | Marimba | *Variety resistant to Chocolate Spot* |
| T | **JOYA** | Joya | *Variety tolerant to Rust* |

### 3.3.2 Chocolate spot (*Botrytis fabae*)

The protocol used is that reported by Villegas-Fernández *et al*. (2012), with minor modifications.

To avoid environmental contamination, a local *B. fabae* isolate was used. The isolate was multiplied in Petri dishes (9 cm diameter) containing potato dextrose agar (PDA)

In order to get the isolate to sporulate, fragments of the PDA medium with the fungi were added to bottles of autoclaved V8 medium (300 ml V8 juice, 150 ml carrot centrifugate, 50 ml deionised water and 3 g agar, at pH 5.5), while still liquid. This mixture was then poured into empty Petri dishes and grown for 7 days at 19°C under a cycle of 12 h of darkness and 12 h visible light+near UV radiation, by which time a carpet of sporulating mycelium was clearly visible (Villegas-Fernández *et al*., 2012).

A spore suspension was prepared from the cultures of *B. fabae* in the V8 medium. For this, a glucose solution (1.2% w/v) was poured onto each dish (about 10-15 ml per dish), and the spores were dislodged by scraping the surface of the medium with a needle. The suspension was filtered through two layers of sterile cheesecloth. The spore concentration was adjusted

with the help of a haemocytometer and diluted with the glucose solution until a concentration of $2.8\times10^5$ spores $ml^{-1}$ was reached. Then, Tween-20 was added to the suspension (0.03% v/v).

Three week- old plants (4–6 expanded leaves) were sprayed to run-off with about 1.5 ml spore suspension per plant. Pots were kept overnight in an incubation chamber in the dark at room temperature with a relative

humidity ≥95%. Then they were transferred to the growth chamber at 20°C with a photoperiod of 12 h of visible light where relative humidity was maintained over 90%.

#### *3.3.2.1 Assessment of the disease*

To evaluate the susceptibility of accessions tested to Chocolate spot, was determined the severity of the disease (DS).

Disease severity was assessed as the percentage of the total plant surface covered with chocolate spot lesions; DS was obtained by scoring the percentage of leaf area affected by the disease, in each leaflet of every leaf of the plant separately, then calculated the average of the leaf and finally the whole plant. The assessment was done starting from the lower leaves and proceeding upwards, up to the last fully expanded leaf at the time of inoculation.

In addition, the damage in stems was assessed on a 0–3 scale where:

- 0: no visible infection;
- 1: a few scattered lesions;
- 2: numerous lesions;
- 3: very numerous lesions. (Rhaïem *et al*., 2002)

### 3.3.3. Rust (*Uromyces viciae fabae*)

In the case of Rust, as Chocolate Spot, each inoculum used in the centre is exclusively collected locally and multiplied every year. The

multiplication of rust inoculum is done *in vivo*, on plants of a susceptible line, inoculated and then grown in growth chambers. At the appearance of the pustules of the disease, the spores are collected in test tubes using a specific aspirator (figure 3.4). The inoculum is stored at -80 °C, in test tubes.

**Fig. 3.4 Aspirator for the collection of urediniospores of rust.**

For the inoculation, at the stadium of 4-6 leaves, 2 mg/plant of freshly collected rust urediniospores of a local of *U. viciae-fabae* mixed with talc powder (1:9) were applied with a blower. Plants were incubated overnight in an incubation chamber, in the dark, at ≥95% relative humidity, and then moved in a growth chamber at 20 °C under a photoperiod at 12 h light and12 h dark.

#### *3.3.3.1. Assessment of the disease*

To evaluate the susceptibility of accessions tested to Rust, the severity of the disease (DS) was determined.

DS was assessed as the percentage of the total plant surface covered with chocolate spot lesions.

It has also been scored the Infection type (IT) according a 0 to 4 scale by Stakman *et al*. (1962) where:

- 0: No lesion;
- 1- 2: A chlorotic spot with a necrotic area in the centre;
- 3: Pustule with a chlorotic circle;
- 4: Pustule with no chlorosis.

Values from 0 to 2 are considered resistant, and from 3 to 4, susceptible.

### 3.3.4. Statistical Analysis

An Analyses of variance (ANOVA) for DS was carried out to evaluate the response of the accessions to the disease. For the analysis, each independent experiment has been considered as a block, with three replicates per block. Statistical analyses were performed using *Statistix10* (Analytical Software, Tallahassee, FL, USA) and *JMP 8* software (SAS Campus Drive, Cary, USA).

## 3.4 Results

### 3.4.1 Chocolate spot

The first lesions appeared 24 h post-inoculation. After 5 days, the symptoms were completely visible and we proceeded to the evaluation. The ANOVA showed significant differences for variable Line (p value equal to 0,000).

The averages of the values of DS and the value of the damage on the stems of both the experiments are reported in Table 3.2.

The general mean of DS the tests is 25.16%. The Standard deviation is 14.60, and the Standard Error is 11.15.

The range of DS values goes from 3.13% of the less susceptible line (the resistant control, Marimba), to 59.47% of the most susceptible lines.

Two TW inbred lines, IS42 and IS46, and a TW synthetic, were found to be more susceptible to *Botrytis* than the susceptible control, Baraka.

The genotypes with good response to Botrytis resulted to be, after Marimba: IS28, IS156 and IS89 and, to a lesser extent, Rumbo, IS48 and STL.

Regarding the values on the stem lesions, the average of the results is 1.40 and the Standard deviation is 0.28.

**Tab.3.2 The average DS (%) and value of the damage on the stem (scale 0-3) for the tested lines for Chocolate spot. In red, the susceptible control, and in green the resistant one.**

| Line | DS (%) | Stem lesions (0-3) | Description |
|---|---|---|---|
| **MARIMBA** | **3,13** | **0,83** | **Resistant test** |
| **IS28** | **8,36** | 0,83 | TW Inbred |
| **IS156** | **10,97** | 1,58 | TW Inbred |
| **IS89** | **12,57** | 1,42 | Inbred |
| **CTL** | **14,19** | 1,67 | Commercial variety |
| **IS48** | **14,37** | 1,13 | TW Inbred |
| **RUMBO** | **14,92** | 1,75 | Commercial variety |
| **STL** | **15,75** | 1,42 | Commercial variety |
| **IS-S5** | **21,74** | 1,75 | TW Synthetic |
| **IS-S4** | **23,26** | 1,42 | TW Synthetic |
| **IS56** | **24,50** | 1,42 | TW Inbred |
| **IS54** | **29,28** | 1,67 | TW Inbred |
| **IS-S6** | **29,75** | 1,17 | TW Synthetic |
| **IS18** | **33,69** | 1,67 | Inbred |
| **IS80** | **37,52** | 1,50 | TW Inbred |
| **BK** | **39,33** | **1,33** | **Susceptible test** |
| **IS-S7** | **39,54** | 1,67 | TW Synthetic |
| **IS46** | **45,64** | 1,33 | TW Inbred |
| **IS42** | **59,47** | 1,13 | TW Inbred |

We performed a Pearson test in order to identify a possible correlation between DS and the values of the lesions on the stem. The results show that there is some correlation, though very weak (see figure 3.5).

Correlations (Pearson)

| | D | STEM |
|---|---|---|
| D | 1,0000 | |
| p-value | 0,0000 | |
| STEM | 0,2307 | 1,0000 |
| | 0,0185 | 0,0000 |

**Fig. 3.5 Pearson matrix of the correlation between DS and Stem lesions.**

We have noted a great variability between the different repetitions of the same genotype, although some accessions have more consistent and stabile response.

### 3.4.2 Rust

The first lesions appeared after 7 days post-inoculation, with visible white spots. After 12 days, the pustules were fully developed and we proceeded to the evaluation.

The ANOVA showed significant differences for variable Line (p value equal to 0,000). The averages of the values of DS for Rust are reported in Table 3.3.

**Tab. 3.3 The Average DS (%) for the tested lines for Rust. In red, the susceptible control, and in green the resistant one.**

| Line | DS (%) | Description |
|---|---|---|
| **JOYA** | **5,15** | Tolerant test |
| **IS28** | **35,83** | TW Inbred |
| **IS80** | **36,48** | TW Inbred |
| **IS89** | **38,31** | Inbred |
| **IS48** | **40,97** | TW Inbred |
| **IS-S4** | **45,00** | TW Synthetic |
| **CTL** | **52,67** | Commercial Variety |
| **IS54** | **57,50** | TW Inbred |
| **IS-S6** | **59,50** | TW Synthetic |
| **RUMBO** | **59,67** | Commercial Variety |
| **IS56** | **61,09** | TW Inbred |
| **IS46** | **63,33** | TW Inbred |
| **IS156** | **63,89** | Synthetic |
| **STL** | **64,67** | Commercial Variety |
| **IS-S5** | **65,00** | TW Synthetic |
| **IS42** | **65,77** | TW Inbred |
| **IS18** | **67,50** | Inbred |
| **IS-S7** | **68,72** | TW Synthetic |
| **BK** | **74,50** | Susceptible test |

The general mean of DS the tests is 53.97%. The Standard deviation is 16.70, and the Standard Error is 6.07.

The range of DS values goes from 5.14% of the less susceptible line (the resistant control, Joya), to 74.50% of the most susceptible lines (the susceptible control, Baraka).

As shown in the table 3.3, none of the tested line is similar, in terms of response, to Joya: this is due to the fact that the resistance of Joya is actually a hypersensitive resistance, that is that the plant has symptoms of the disease but are controlled. Joya has a type of infection (IT), according to the scale by Stackman *et al*. (1962) 1-2, instead each of the other lines, has a type of infection white flower, white seed coat and white hilum

## 3.5 Discussion and Conclusions

The inbred lines that were chosen to be tested for the resistance to Rust and Chocolate spot, are among the most interesting lines of the population from an agronomic point of view. Unfortunately, it was not possible to test all the Synthetic lines currently under evaluation, as we wished, for a problem with availability of seeds of IS-Sint2. IS-Sint1 and IS-Sint3 were discarded from further evaluation, following the preliminary agronomic evaluation, which gave very negative results in terms of yield.

The tests conducted on the selected lines have obtained interesting results, in particular for Chocolate spot. The differences of response to *Botrytis fabae* between genotypes are evident and very clear. Accessions showing a good level of resistance to Botrytis, consistent and stable have been identified.

The great variability found between replicas of the same accession, can be explained by two factors:

- In the case of Synthetics and Cultivars: due to the partially allogamous nature of *Vicia faba* (Bond and Poulsen, 1983), within these two types of material there is a high degree of variability. In the case of faba bean, in fact, there are populations in which, at the same time there are self-fertilized plants, along with plants subject to cross-pollination by

pollinators. The degree of heterozygosity is very high, and may explain the different degree of response to disease.

- Differences in the distribution of the inoculums, or influence of the position in the incubation chamber and/or growth rooms on the response, especially for the degree of relative humidity. This is very common in the case of these foliar necrotrophic diseases, and increasing the number of replications and/or experiments is recommended.

The two lines, after Marimba, which show a degree of relevant low resistance to Chocolate Spot, are two triple-white inbred lines. A result to emphasize because,

- those TW lines can be used for breeding purposes;

- it is another demonstration of the fact that the triple-white lines are not, as a rule, more susceptible to disease, as considered by Helsper *et al.* (1994) and already refuted by Cubero and Duc (2005).

The relationship between the damage in stems and in leaves is very weak, and it could be due to chance. A good example for this is the case of the three accessions that have given the best results in terms of DS: only one, IS28, has a low value of the stem infection, while even IS156 has a high value of infection, above the average of the test. Rhaïem *et al*. (2002) and Villegas-Fernandez *et al.* (2012), have observed that there is no correlation between these two values.

It is therefore advisable, in the selection for breeding for Chocolate Spot, consider only the value of DS on leaves

The results relating to Rust, revealed that there are not resistant lines among the population tested, comparable to the resistant control. The mechanism of resistance of Joya to *U. vicia-fabae* is of hypersensitive type, described for the first time in faba bean by Sillero *et al*. (2000). This defence mechanism causes the infection of the plant takes place, but the type of infection (IT) is not very harmful to the plant. The key mechanism of this hypersensitive response is the death of cells infected by the fungus, so further development of the pathogen is hampered. In the scale by Stackman *et al*. (1962), hypersensitivity corresponds to a value of IT from 1 to a maximum of 2, with necrotic spots surrounded or not, by a chlorotic halo.

Several sources of polygenic quantitative resistance against *U. viciae-fabae* have been reported in faba bean in the last decades, but, along with the hypersensitive response, both types bring an incomplete resistance, associated with an increased latent period, a reduction in colony size and a decreased infection frequency.

Some lines have shown, however, a relatively low DS value, even if with high value of IT, and can be considered a good basis for breeding strategies.

An important result of this work, looking at the data, is that three inbred lines, IS28, IS48 (both TW) and IS89 with a good response to both diseases have been identified. In particular, IS28 was found to be the best line in both the tests. This is a very important result for breeding, because of the saving in time and in economic terms that means to have a multiple-disease resistance (Villegas-Fernández *et al*., 2011). Although a number of sources of resistance to both diseases have been already identified (Tivoli *et al*., 2006; Villegas-Fernández *et al*., 2009; Sillero *et al*., 2010), it needs a lot of work to transfer both resistances into advanced inbred varieties, in order to obtain commercial varieties.

The best of the TW Synthetic lines tested it is resulted to be IS-Sint4, although with performances far below of the above mentioned inbred lines. It is interesting to observe that in the composition of IS-Sint4, there is the IS28.

On the other hand, it is disappointing the performance of IS18, a promising inbred line from the agronomic point of view, which instead was found to be very susceptible to both pathogens.

For the future, the lines should be also evaluated in the field, where the pressure of the disease is lower, but the environmental conditions are more unstable, to confirm the resistance data from controlled environment.

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## *Final considerations and future perspectives*

The main challenge in performing this work, has been the availability of resources. The low yield of this faba bean species, made even lower by the forced self-pollination (*Vicia faba* has a reproductive system only partially self-fertile) and the limited availability of seed, have limited the number and size of the tests carried out.
The Augmented Block Design test to assets the agronomic performance of the lines, was repeated for a single year as the availability of seed has not allowed us to repeat in the following years. In fact, to observe its agronomic value, the lines are left to free pollination, and consequently the seed obtained cannot be reused.

The availability of seeds, in addition, together with the phenotypic similarity of the seed and the plant, has been the main factor taken into account for the selection of 15 lines that form the 7 combinations of synthetic lines tested preliminarily in this work.

The stability of the yield is one of the main problems of the cultivation of *Vicia faba*, and indeed, our choice to create synthetic varieties was also made in order to exploit the heterosis factor, while maintaining the characteristics, phenotypic and qualitative, significant from the point of commercial view: Triple White (white flower, seed coat and hilum, traits associated with a low level of anti-nutritional compounds) and an

high level of self-fertility (which guarantees a certain level of yield, even in case of little presence of pollinating insects).

Aware that a molecular-genetic characterization, according to the most innovative techniques available today, allows us to speed up and deepen this work of characterization of our material, we started a parallel work of genotypic characterization of the material, which it is currently at an early stage.

For this purpose, in collaboration with IGA (Institute of Applied Genomics), we developed a protocol for obtaining dd-RAD markers on *V. faba*, starting from the protocol developed by Peterson *et al*. (2012) (Nanni *et al*., 2015). The lines that we have chosen for the development of the protocol, are two parental of the population, *Bachella* and *Larga di Leonforte*, and a Triple White (TW) inbred line, *IS48*, selected for good agronomic value. The purpose was to obtain primers designed specifically for our population, to achieve a surely result, which could not be certain if we had used one of the set of SNPs currently available for faba bean (see rewiev by O'Sullivan and Angra, 2016).
Filtering the positions obtained with the GbS method, we obtained a set of 1776 SNP markers. As we got a very large set of SNPs, we will proceed to select a little set by comparison with sequences of other related species and not related to *Vicia faba*, already present in genebanks. Then we will proceed with the validation of the markers,

and we will use the SNPs obtained to study correlation between phenotype and genotype in our population, and to investigate the variability of a collection of V. faba from various areas of the world. Being the three lines very different (*Bachella*: coloured variety, small seeds and high self-fertile; *Larga di Leonforte*: coloured variety, very large seeds; *IS48*, TW inbred) which have been chosen for obtaining SNPs, the set of markers obtained could be useful for the characterization of the species. We also initiated the creation of a segregating population, through crossing the same three lines used for the GbS work, in order to obtain useful material for the creation of a new genetic map.

With this work, therefore, we want to contribute to the global knowledge on *Vicia faba*, as well as create new commercial materials that could be a viable alternative to existing varieties, with a focus on yield stability and low content of anti-nutritional factors, the main breeding objectives for this species.

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